CHARACTERIZATION OF THE ANTIAGING EFFECTS OF APPLE PHYTOCHEMICALS USING THE *C. ELEGANS* MODEL

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Aging is a major risk factor for many chronic diseases, including cancer and cardiovascular disease. The resulting need to understand and alleviate age-related decline has led to pharmacological manipulation of age-related degeneration in many species, including the nematode *Caenorhabditis elegans*, one of the simplest whole-animal models. However, few such studies have examined the anti-aging effects of natural compounds found in fruits and vegetables.

Apples are the number one source of phenolics in U.S. diets. Apple phytochemicals have been shown to have a variety of salubrious effects, including high antioxidant and antiproliferative activity and the modulation of several molecular targets. We treated *C. elegans* with apple extracts, and found that lifespan increased significantly by up to 39%. Healthspan was also significantly improved as measured by two biomarkers, lipofuscin and motility. Additionally, animals pre-treated with apple extracts were more resistant to stresses such as heat, UV radiation, paraquat-induced oxidative stress, and pathogenic infection, suggesting that cellular defense system functions were also improved. To find the mechanisms behind these improvements, we screened several mutants that affect lifespan via different pathways. Prolonged lifespan does not appear to be mediated by calorie restriction, insulin signaling, sirtuins, or germ line signaling. Instead, the effects are at least partly due to reduced oxidative stress, as indicated by the increased

lifespan of the *mev-1* mutant. We also observed an increased induction of the promoter activity of small heat shock protein *hsp16.2*, and a reduction of induction of the promoter activity of superoxide dismutase and glutathione-S-transferase, two enzymes that attenuate excessive reactive oxidative species. This is consistent with the idea that apple extracts may protect against deleterious oxidative damage normally mitigated by *sod-3* and *gst-4,* leading to a decreased need for this part of the endogenous antioxidant system.

We propose that in *C. elegans* apple phytochemicals act through a synergistic mechanism that involves attenuation of ROS damage, in particular superoxide anions, and generalized detoxification of oxidation byproducts. In addition, increased induction of the promoter of *hsp16.2* likely contributes to a general improvement of cellular detoxification and stress response, and explains improved survival under conditions of stress.

BIOGRAPHICAL SKETCH

Elena Markusovna Vayndorf was born in Odessa, Ukraine. She immigrated to the United States with her family at the age of 11 and settled in the Bronx, NY where she attended public schools, including the competitive Bronx High School of Science. Elena enrolled in Hunter College, City College of New York, and after two years transferred to Cornell University where she completed her Bachelor's of Science degree in Biological Sciences, with a minor concentration in Modern European Studies.

After a two-year stint in industry, Elena returned to Cornell to attend graduate school in the Field of Environmental Toxicology, with minors in Risk Assessment and Nutrition. She joined the laboratory of Dr. Rui Hai Liu, where she developed a project that combined her major research interests in the biology of aging, health benefits of natural products, and nutrition. During her graduate career, she also worked as a teaching assistant, served as student representative to the Curriculum Committee of the Field of Environmental Toxicology, and was a member of the Association of Comparative and Environmental Toxicology Students (ACETS).

While working toward her degree, Elena also pursued a number extracurricular activities, including fundraising for, shooting, editing and screening a documentary about Russia, showcasing a photography exhibit at a local art gallery, and leading a student group as a Native Speaker in the Language House program. In addition, she kept herself busy by taking up swing dancing, recreational swimming, and attending an embarrassingly large number of seminars, talks, concerts and festivals on and off campus.

To my parents, for their unconditional love, support and encouragement

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LIST OF ABBREVIATIONS

CVD	Cardiovascular disease
AE	Apple extracts
AH	Antioxidant
AHR	Aryl hydrocarbon receptor
CYP	Cytochrome P450
EGCG	Epigallocatechin gallate
HepG2	Hepatocellular carcinoma cells
HL-60	Human promyelocytic leukemia cells
FUDR	5-Fluoro-2-deoxyuridine
GFP	Green fluorescent protein
IGF	Insulin growth factor
IIS	Insulin/IGF signaling
LDL	Low-density lipoprotein
LB	Luria broth
MAPK	Mitogen activated protein kinase
NGM	Nematode growth medium
OS	Oxidative Stress
PAHs	Polycyclic aromatic hydrocarbons
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
ROS	Reactive oxygen species
RNAi	RNA interference
RNS	Reactive nitrogen species
TOR	Target of rapamycin

CHAPTER 1

Introduction: Health impacts of phytochemicals

Epidemiological studies have consistently shown a wide variety of health benefits associated with a diet rich in plant foods, especially fruits, vegetables and whole grains (1-3). Phytochemicals, the bioactive plant compounds that are not essential for human nutrition, are believed to be responsible for many of these health benefits, including the prevention or treatment of chronic diseases (4). Besides normal upkeep and maintenance, plants produce many of these bioactive compounds to defend themselves against microbes and insects, and to ward off diseases. To date, more than 5000 phytochemicals have been identified, and many remain unknown (5). The most common sources of phytochemicals in foods include fruits, vegetables, and grains (4).

Phytochemicals can be classified into five major groups: phenolics, carotenoids, alkaloids, nitrogen-containing compounds and organosulphur compounds. The phenolics can be further subdivided into phenolic acids, flavonoids, stilbenes, coumarins and tannins. Flavonoids can be divided still further. Importantly, the flavonols and flavanols (catechins) are found in fruits and are known to have a wide variety of health benefits. Most notably, these compounds have been associated with a reduced risk of developing chronic diseases such as cancer, cardiovascular disease (CVD), diabetes, Alzheimer's disease, cataracts and other age-associated maladies (6-9).

Phytochemicals possess a wide range of biological activities. These include antioxidant activity (e.g. scavenging of free radicals), inhibition of cell proliferation (e.g. inhibition of HepG2 liver cancer cells by apple extracts), inhibition of cell differentiation (e.g. arrest of MCF-7 breast cancer cells in G1/

S phase by apple extracts), inhibition of oncogenes (e.g. inhibition of AP-1 tumor promotion protein by apple extracts) and induction of phase II enzymes (e.g. the induction of glutathione by sulforaphane, a phytochemical found in cruciferous vegetables such as broccoli) (10-13).

1.1.1 Evidence for prevention of cancer

Cancer is a group of diseases in which cells display uncontrolled growth, invasion of adjacent tissues, and sometimes metastasis. Carcinogenesis is a three-step process consisting of initiation, promotion and progression. During initiation, DNA undergoes damage by internal or external factors such as genomic instability and excessive reactive oxygen species (ROS) from cigarette smoke, UV radiation from the sun or polycyclic aromatic hydrocarbons (PAHs) from grilled or charbroiled foods (14). If the damage in the mutated DNA is not repaired, when the cell divides it multiplies along with the mutated DNA and the process enters the promotion stage, which is the clonal proliferation of mutated cells. During the third and final stage of cancer promotion, the mutated DNA affects the genes controlling proliferation, survival, which leads to malignancy. The first two stages, initiation and progression, are reversible, but the third stage, progression, is not. Once the cells become malignant, they can spread to other parts of the body and take over surrounding tissues in a process called metastasis. This is the deadliest aspect of the disease.

Because cancerous tumors develop and sustain themselves by many different mechanisms, a variety of chemopreventative and chemotherapeutic approaches have been used. Among these are dietary phytochemical interventions whose role in chemoprevention has been well documented (10).

Block *et al.* reviewed close to 200 studies that examined the relationship between cancer risk and consumption of fruits and vegetables. In particular, the authors looked at cancer of the breast, ovary, colon, lung, cervix, esophagus, stomach, oral cavity, bladder and pancreas. In 128 of 156 studies, the authors found a significant protective effect with increased consumption of fruits and vegetables. In persons with a low consumption of fruits and vegetables, the risk of developing cancer increased by two-fold compared to those who consumed a high amount (15). Another prospective study looked at the flavonoid consumption of nearly 10,000 Finnish men and women and found that the risk of cancer incidence was inversely related to the number of fruits and vegetables consumed by the study participants (16). Of the major dietary flavonoids, those found in apples provided the greatest protection against lung cancer. In a population-based case-control study of women in Shanghai, pre-menopausal women who ate more dark yellow-orange vegetables and citrus fruits had lower incidence of breast cancer than those women who did not (17). Dietary phytochemicals have been shown to exert their chemopreventative effects by a variety of mechanisms. For example, they may affect DNA repair, carcinogen metabolism, cell proliferation, apoptosis, inflammation and antioxidation (10). To date, more than 1000 phytochemicals have been shown to have chemopreventative properties (18). The mechanisms are cell-type or dose-dependent and often involve more than one target. For example, Epigallocatechin gallate (EGCG), a compound found in green tea, can inhibit carcinogen activation and DNA binding, as well as decrease CYP protein expression and AHR binding to DNA in HepG2 liver cells. Lycopene, a compound found in tomatoes and tomato products, was shown to influence the cell cycle by decreasing cyclin D1 and D3 expression

in human breast and endometrial cells, and increasing apoptosis in HL-60 cells. Similarly, curcumin, a phytochemical from curry, has been shown to control the cell cycle and cell proliferation by decreasing the expression of cyclin D1 in prostate and breast cancer cells and also decreasing c-Jun and c-Fos expression in mouse epidermal (JB6) cells.

1.1.2 Evidence for prevention of heart disease

Cardiovascular disease (CVD) is the number one cause of death and disability in the United States and around the world. CVD is a group of diseases and includes coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. The major causes of cardiovascular disease are tobacco use, an unhealthy diet and physical inactivity.

Heart attacks and strokes are mainly caused by blockage that prevents blood from flowing to the heart or the brain. The most common reason this occurs is build-up of fatty deposits on the inner walls of the blood vessels that supply blood to these organs. As the blood vessels become narrower and less flexible (atherosclerosis), they are more likely to become blocked by blood clots. As a result, the proper amount of blood is not supplied to the heart and brain, and damage occurs. On a molecular level, excessive oxidation of low-density lipoprotein (LDL) in the plasma and walls of the arteries may lead to their engulfment by macrophages and formation of foam cells. These cells line the arterial walls and lead to the formation of fatty streaks which progress to advanced lesions. The lesions restrict blood flow and lead to the different manifestations of CVD (19).

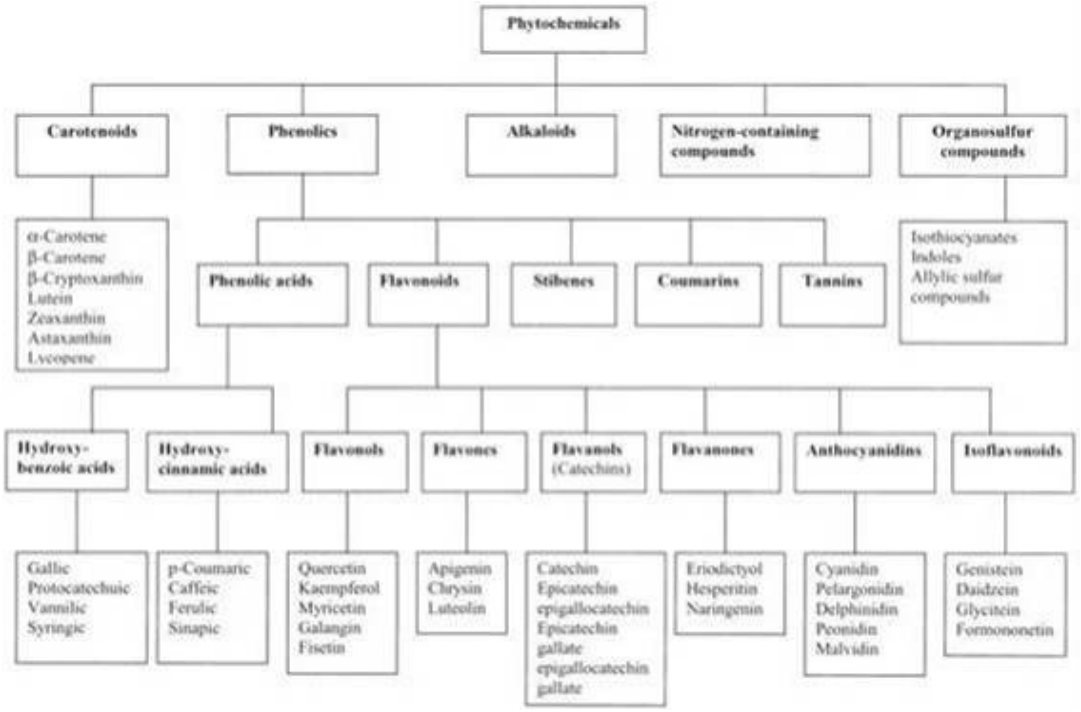


Figure 1.1 Classification of dietary phytochemicals (adapted with permission from Liu, RH) (10).

Numerous studies have suggested that phytochemicals play a role in the prevention of CVD, and diets that are rich in phytochemicals have been associated with a reduction in LDL, cholesterol and mortality from coronary heart disease. For example, dietary flavonoids have been inversely correlated with the risk of developing coronary artery disease and myocardial infarction. A study from Japan showed that flavonoid intake was inversely associated with total plasma cholesterol and low-density lipoprotein (LDL). Similarly, Joshipura and colleagues reported that a total intake of greater than 4 fruits and/or vegetables per day, was associated with a decreased risk of coronary artery disease (2).

Mechanisms for the prevention of arteriosclerosis by phytochemicals with antioxidant properties have been proposed. In the LDL oxidation hypothesis, antioxidants consumed from food are incorporated into LDL and are themselves oxidized when the LDL are exposed to prooxidative conditions. Due to their reduced redox potential, the antioxidants prevent any extensive oxidation that may otherwise occur in the sterol or polyunsaturated fatty acids. Other potential mechanisms by which phytochemicals may prevent CVD include the reduction of platelet aggregation, modulation of cholesterol synthesis and absorption, and reduction of blood pressure. Increased inflammation is a strong predictor of cardiovascular disease (4). It promotes initiation and progression of atherosclerosis, and can also cause acute thrombotic complications that are associated with atherosclerosis. Many phytochemicals have antiinflammatory activity and thus may play an important role in the prevention of cardiovascular disease. For example, quercetin, which is found in apples and onions, has a range of antiinflammatory effects on prostaglandins, leukotrienes, histamine release and subsequent

antiasthmatic activity. Likewise, organosulfur compounds have been shown to have strong antiinflammatory effects. Chang and Chen found that nitric oxide (NO) and prostaglandin E2 (PGE2) production is inhibited by the garlic oil derivatives, diallyl sulfide (DAS), diallyl disulfide (DADS), and allyl methyl sulfide (AMS), in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage cells (20).

1.1.3 Evidence for prevention of diabetes

Type 2 diabetes, also known as *Diabetes mellitus*, is a disease, characterized by high blood glucose levels, in which the body develops resistance to insulin and relative insulin deficiency. Type 2 accounts for 90% of all cases of diabetes. In the United States, there are an estimated 23.6 million people living with the disease (21). At its onset, the disease can be managed by dietary modification and regular exercise, however as it progresses, pharmacological intervention is required.

In a healthy individual under normal conditions, after food is consumed glucose is released into the bloodstream and stimulates the secretion of insulin by the pancreas. Insulin lowers blood-glucose levels by promoting glucose uptake by skeletal muscle and inhibiting hepatic gluconeogenesis. If there is a constant period of food intake, insulin secretion from the pancreas is also increased. This leads to accumulation of triglycerides in adipocytes and over time visceral obesity. In turn, factors such as free fatty acids (FFA) and tumour-necrosis factor-α (TNFα) inhibit insulin-mediated glucose uptake into skeletal muscle and inhibits hepatic gluconeogenesis. To prevent an increase in blood glucose, the pancreas increases insulin secretion, which results in a feed-forward cycle and leads to an altered metabolic state involving visceral

obesity (22). Hyperglycemia (increased blood-glucose) and dyslipidemia (disruption of the amount of fatty acids and lipoproteins in the blood) can induce the inflammatory and oxidative stress responses, which leads to the generation of free radicals (23). In the case of type 2 diabetes, oxidative stress may block the action of insulin by impairing membrane fluidity, decreasing availability of nitric oxide (NO) or increasing intracellular calcium content. Multiple studies have shown that α- and γ-tocopherol, β- and α-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, retinol, as well as ascorbic acid are significantly decreased in diabetic individuals (24-26). Thus, the use of antioxidant therapeutics in the treatment of diabetes has been proposed (23).

Flavonoids, carotenoids, ascorbic acid and tocopherols are the major groups of phytochemicals, which have been recommended due to their antioxidant and other health benefits (23, 27). These phytochemicals have been shown to inhibit free radical producing enzymes such as xanthine oxidase, cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione-S-transferase, mitochondrial succinoxidase, NADH oxidase), chelating trace metals and inhibiting phospholipases A2 and C. The antioxidant mechanism of action is also mediated through donation of a hydrogen atom or electron to the many ROS radicals such as super-oxide anion, and hydroxyl, alkoxyl and peroxyl radicals, which protects lipoproteins, proteins and DNA molecules against oxidative damage. Other mechanisms by which phytochemical antioxidants may exert their effects are gene regulation, differentiation and apoptosis (23).

Besides antioxidant activity, Sun and colleagues have shown that SIRT1, a histone deacytelase, improves insulin sensitivity by repressing *PTP1B,* a phosphatase, which reverses tyrosine kinase activity. The authors showed

that SIRT1 is downregulated in insulin-resistant cells and tissues and that knocking down SIRT1 induces insulin resistance. Moreover, overexpression of SIRT1 led to improved insulin sensitivity, especially under insulin-resistant conditions. Resveratrol, a phytochemical found in grapes and other plant foods, and an activator of SIRT1, was also found to improve insulin sensitivity *in vitro* in a SIRT1 dependent manner. When 2.5 mg/kg/day of resveratrol was administered to mice that were fed a high fat diet (which induces insulin resistance), the animals showed improved insulin sensitivity versus the control. The authors found that this effect was mediated via *PTP1B* at the chromatin level, and concluded that SIRT1 improves insulin sensitivity and could be useful in the treatment of insulin resistance and type 2 diabetes (28).

1.1.4 Evidence for prevention of Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia in the elderly in the United States, and affects over 26 million people around the world (29). The clinical symptoms begin with memory impairment and progress to severe dementia. This process is brought about by the selective degeneration of nerve cells in the brain regions that are responsible for memory, cognitive performance and personality. The disease is characterized by the accumulation of amyloid beta peptide ($A\beta_{1-42}$) aggregates and an increase in oxidative stress. One of the major hypotheses of AD is that the accumulation of amyloid beta deposits leads to downstream neurotoxic events, such as increased oxidative stress, which lead to neuronal dysfunction and death (30). Aksenov *et al.* suggested that protein oxidation is the major contributor to AD-associated neuron death and decline of cognitive abilities. Specifically, the oxidation of amino acid residues in proteins was suggested to

lead to loss of protein function (31). However, protein oxidation may not represent the whole picture. Schukitt-Hale and colleagues have shown that antioxidation is only one among many properties that predicted whether a particular plant food improved age-related changes in the brain . In one study, the authors fed rats with spinach, strawberry or blueberry supplemented diets, all of which had the same amount of antioxidant activity (as determined by the ORAC assay), and assessed the animals for cognitive behavior markers. The blueberry diet showed the greatest increases in motor performance, carbachol-stimulated GTPase activity, oxotremorine-enhanced DA release, and calcium recovery following exposure to oxidative stress. Since all of the extract-supplemented diets had the same amount of antioxidant activity, the authors concluded that antioxidation must not be the only factor that determines neuroprotective, anti-aging properties of phytochemicals. In a follow-up review, this group proposed that besides lowering oxidative stress, berry fruits could slow brain aging by lowering inflammation, modulating cell signaling involved in neuronal communication and neuroprotective stress shock proteins, and buffering calcium levels (33). Similarly, Casadesus and colleagues found that a diet supplemented with blueberries improved spatial memory and increased parameters associated with hippocampal neuronal plasticity, which correlates with improvements in spatial memory and cognitive performance (34). The authors examined a number of markers of hippocampal plasticity, including extracellular receptor kinase activation, and IGF-1 and IGF1-R levels in blueberry-supplemented aged animals. The results showed that all parameters were increased in supplemented animals. In addition, work done on *C. elegans* has shown that the soy isoflavone glycitein protects against beta amyloid-induced toxicity and oxidative stress (35). Transgenic

animals expressing the human amyloid beta peptide ($A\beta_{1–42}$) were fed a diet supplemented with glycitein. These had reduced amounts of beta amyloid and hydrogen peroxide. The authors concluded that this isoflavone suppresses Aβ activity through antioxidant activity as well as inhibition of Aβ deposits.

1.1.5 Evidence for prevention of eye aging

The eye also undergoes age-associated deterioration, which often manifests itself in diseases such as macular degeneration, glaucoma, cataracts and vascular degeneration. Ocular structure, function and blood flow all markedly deteriorate with age, as does visual acuity, contrast sensitivity and dark adaptation threshold (9). Moreover, there is a loss of retinal pigment epithelial cells and photoreceptors (36). Other pathophysiological changes with age include a decrease in the number of axons, a decline in retinal nerve fibers (which represents a loss of about 60,000 retinal ganglion cells per decade) and an increase in the susceptibility to damage to the retinal ganglion (9). In addition, during both aging and glaucoma, individuals experience a number of common symptoms including blood flow abnormalities, narrowing of blood vessels and endothelial dysfunction (9).

Age-related macular degeneration is linked to oxidative stress, which is believed to be one of the mechanisms responsible for the aging changes in retinal pigment epithelial (RPE) cells. Lipofuscin, the photoreactive pigment that accumulates with age in many tissues, including eyes, contributes most of this oxidative damage to RPE cells. A recent study by Zhou *et al.* has shown that administration of sulforaphane, a phytochemical that induces phase 2 enzymes to RPE cells, indirectly decreased oxidative damage and elevated levels of NAD(P)H:quinone reductase, and glutathione-S-transferases, phase

2 enzymes in these cells. The authors concluded that, in addition to carotenoids and antioxidant vitamins, plant-derived phase 2 inducers such as sulforaphane may also be important in the prevention or treatment of age-related macular degeneration (37).

1.2 Classification and characterization of apple phytochemicals

Apples are the second most highly consumed fruit (after bananas) in the United States and the number one source of phenolics in American diets (38, 39). According to the U.S. Department of Agriculture, the average adult consumes approximately 16 pounds of fresh apples per year. The health benefits of apples are in large part due to their unique phytochemical profiles. In particular, apples are a good source of phenolics compounds. Of these, the vast majority are in the flavonoids group (40). The total phenolic content has been estimated to range from 110 to 357 mg/100 g fresh apple (9, 10). Tsao *et al.* have reported that phenolics are five times more prevalent in the skin than the flesh of the apples (41). The nature and distribution of these compounds between the skin and the flesh is also different. Whereas the flesh contains catechins, procyanidins, phloridzin, phloretin, the skin has additional flavonoids such as quercetin glycosides and cyanidin glycosides (42).

Among these compounds, several classes of phenolic antioxidants have been described. These include: cinnamates, flavan-3-ols, flavonols, procyanidins, dihydrochalcones, and anthocyanins (43).

Table 1.1 Antioxidant activity of different apple varieties (adapted from Tsao *et al.*) (41)).

Apple Variety	Antioxidant Activity (Peel/Flesh) (μM Vit. C Equiv.)	Phenolic Content (Peel/Flesh) (μg/G)
Red Delicious	4,112±13/2,192±4	2,012±20/358±12
Northern Spy	10,044±20/5,049±1	1,548±24/934±7
Ida Red	5,958±17/3,069±0	1,479±21/489±1
Cortland	4,133±16/1,629±1	1,323±21/498±11
McIntosh	5,531±23/2,967±1	1,163±17/488±10
Golden Delcious	5,223±11/2,192±4	1,265±22/417±15
Mutsu	4,643±24/1,895±14	1,017±26/313±13
Empire	3,800±4/1,457±3	782±14/177±1

Recently, research from our laboratory has identified 29 structurally diverse compounds from apple peels, many of them unique, using bio-guided fractionation. The most prominent were quercetin-3-O-β-D-glucopyranoside, quercetin-3-O-β-D-galactoupyranoside, quercetin, catechin, epicatechin and quercetin-3-O-α-L-arabinofuranoside (40).

Tsao *et al.* found that, of the major polyphenols that they examined, two compounds, epicatechin and procyanidin B2, were the greatest contributors to total antioxidant activity. Of the eight varieties of apples examined, the Red Delicious variety had the highest antioxidant activity (41). In addition, procyanidins made up approximately 60 percent of the antioxidant activity in the peel and 56 percent in the flesh. The researchers concluded that: "When taste and texture do not matter, choosing an apple with a high proportion of polyphenols in the flesh and skin can potentially produce more-health benefits," and also that: "eating any apple is better than eating no apple at all."

The present study used the Red Delicious variety of apples for a number of reasons. First, this variety of apples is the most widely consumed in the U.S. and accounts for 27% of U.S. apple production (44). This variety also had the highest level of antioxidant activity among eight popular varieties of apples as shown above (41). Additionally, the effects and health benefits of Red Delicious apple phytochemicals have been well characterized in our laboratory (see discussion below). Overall, apples possess a wide range of biological health-promoting activities (38).

1.3 Absorption and metabolism of flavonoids

Flavonoids from apples are found mainly in the glycosylated form, attached to a sugar moiety. In humans as well as rats, upon entering the oral

cavity these compounds undergo hydrolysis and then are further hydrolyzed in the small intestine. The flavonoids such as quercetin are absorbed as aglycones. The vast majority of absorption occurs in the small intestine and colon through the microflora. The human intake of flavonoids is about 300-650 mg/day. In terms of metabolism, a variety of phase I, p450 CYP enzymes are responsible for adding functional groups and activating flavonoids in preparation for uptake or excretion. For example, CYP1A2 as well as CYP2E1 can efficiently oxidize several isoflavones. Whereas, phase II enzymes such as glucoronide sulfatase, as well as the bacterial enzymes UDP-glucuronosyltransferase and sulfotransferase can convert the activated phytochemicals to flavonoid glucuronide and flavonoid sulfate. Other important players in the absorption of flavonoids are the sodium-dependent glucose transporter 1 (SGLT1) and multidrug resistance-associated proteins 2 and 3 (MRP2 and MRP3), which are membrane transporters (45).

Based on direct evidence, Hollman *et al.* have proposed that flavonoid glycosides actually could be absorbed intact in the small intestine, using the sodium-dependent glucose transporter (SGLT1) (46). On the other hand, it was shown that the efficiency of this absorption was suppressed by efflux of some of the flavonoid glycosides shown that by the apical transporter multidrug resistance-associated protein 2 (MRP2) (45). In addition, other evidence has shown that some flavonoid glucosides can be hydrolyzed in the oral cavity small intestine (47).

1.4 Effects of apple phytochemicals: evidence from chemistry assays

The antioxidant activity of apples has been well established in a variety of chemistry assays. Using the ABTS assay, Vieira *et al.* have shown that

within a variety of cultivars tested, the total phenolic, flavanol and anthocyanin contents and antioxidant activity were highest in the peels, followed by the whole fruit and the flesh. Total phenolic content was correlated strongly with total antioxidant activity in flesh, whole fruit and peel. The researchers concluded that phenolic compounds contribute a significant amount of antioxidant activity, and that this activity varies considerably depending of the part of the apple and cultivar analyzed (48). Similarly, Tsao *et al.* used a PCL assay to show that among the eight most popular apple varieties in the U.S., Red Delicious apples have the highest total antioxidant activity. The authors also found that apple peels have significantly more phenolics than does the flesh as was previously reported. Of the major phenolic compounds that were tested, epicatechin and procyanidin B2 contributed the highest antioxidant activity (41).

1.5 Effects of apple phytochemicals: evidence from cell culture assays

Cell culture experiments have demonstrated that apples possess strong antioxidant and antiproliferative activities. Studies from our laboratory have shown that apple extracts have potent antiproliferative activity against HepG2 liver cancer cells and Caco-2 colon cancer cells (49). Notably, apples with peels had considerably more activity than apples without. In addition, the extracts also had strong antioxidant activity using the TOSC assay. Interestingly, Vitamin C contributed only a small percentage of this activity. In a follow-up study, Sun *et al.* surveyed 11 of the most commonly consumed fruits and found that apples had the second highest total antioxidant activity behind cranberries (50). Similarly, Wolfe *et al.* showed that among 25 of the most commonly consumed fruits, apples have the highest antioxidant activity

and are the number one contributor of phenolics (39). Others reports have also confirmed that apple phytochemicals possess potent antioxidant and antiproliferative activities *in vitro*. For example, He and Liu have shown that 2-α-hydroxy-ursolic acid, one of the phenolic acids found in apple peels, has potent antioxidant and antiproliferative activity (51). In a follow-up report, these researchers isolated additional compounds from apple peels and showed that the flavonoids quercetin and quercetin-3-*O*-β-D-glucopyranoside had the highest antioxidant and antiproliferative activity among 29 structurally diverse compounds (40). 2-α-hydroxy-ursolic acid has also been shown to inhibit TNF-α induced activation of NF-κB in MCF-7 cells in a dose dependent manner (52). Additionally, Red Delicious apple extracts have been shown to induce G1 arrest and decrease the expression of Cyclin D1 and Cd4, and inhibit the tumor activator protein AP-1 (11, 12). Another study looked at the effects of apples, bananas and oranges on oxidative stress-induced damage to neuron like PC12 cells and found that these cells were protected against neurotoxicity after they were exposed to hydrogen peroxide treatment (53).

1.6 Effects of apple phytochemicals: evidence from animal studies

Data from animal studies have confirmed that apple phytochemicals have potent anticancer effects *in vivo.* A study by Liu *et al.* showed that consuming the human equivalent of 1, 3 and 6 apples per day reduced DMBA-induced incidence of mammary tumors in rats by 17, 39 and 44% respectively (54).

In a follow-up study, rats were administered whole apple extracts 2 weeks prior to DMBA administration and continuing for 24 weeks. The animals developed mammary tumors with an incidence of 71.4%. In contrast, animals

that were pretreated with apple extracts showed a significantly reduced burden of mammary tumors in a dose dependent manner. In particular, the number of adenocarcinoma masses decreased with increasing apple extracts. In addition, the expression of proliferating cell nuclear antigen (PCNA), cyclin D1, and Bcl-2 decreased, and Bax expression and apoptosis all increased with increasing apple extracts (55).

1.7 Effects of apple phytochemicals: evidence from epidemiology

Epidemiological studies have shown a correlation between the intake of fruits and vegetables and the incidences of cancer and cardiovascular disease (2, 15). Several studies have looked at apples or apple phenolics in particular. A few of these are reviewed below.

In a recent retrospective case-control study, Jedrychowski and colleagues have shown that apple consumption is associated with a reduced risk of colorectal cancer (56). Colorectal or colon cancer is the third most prevalent cancer and the second most lethal cancer among all cancers in the Western World. The authors interviewed a total of 186 colorectal cancer patients for whom information on histology, anatomic location and stage of cancer was available, and compared this with 211 noncancer controls from the same hospital taking into account confounding factors such as age, gender, place of residence, marital status and occupation. It was found that patients who consumed at least one apple per day had a reduced risk of developing colon cancer. Fruits such as citrus fruits and berries did not show such a correlation.

Le Marchand *et al.* conducted a population-based retrospective case-control study in Hawaii to look at the effect of diet on incidence of lung cancer

(57). In an in-person interview, the researchers assessed smoking history and intake of 242 food items for 582 patients with lung cancer and 582 controls who were matched for ethnicity, age, and gender. After adjusting for smoking and intakes of saturated fat and ß-carotene, it was found that there was a statistically significant inverse association between lung cancer risk and the major food sources of two flavonoids: quercetin, which is present in apples and onions, and naringin, which is present in white grapefruit. Interestingly, the authors found that squamous cell carcinoma was strongly modified by the CYP1A1 genotype and speculated that this might be due to the inhibitory properties of quercetin in subjects who consumed flavonoid-rich foods, as was reported previously.

Sesso and colleagues examined the risk of flavonoid consumption and risk of cardiovascular disease (CVD) in women. 38,445 women were enrolled in a prospective study with a mean follow-up of 6.9 years. The researchers distributed a food-frequency questionnaire and analyzed the amounts of flavonoid consumption based on the reported flavonoid-rich food content. The mean flavonoid intake was determined to be 24.6 ± 18.5 mg/d, primarily as quercetin (70.2%). It was found that there was no significant difference between intake of flavonoids and CVD. It is worth pointing out that, both broccoli and apples were associated with a lower risk of CVD; however, these differences were not statistically significant.

The conclusion from this study was that flavonoid intake is not strongly associated with a reduced risk of CVD. Moreover, there were no immediate inverse associations for broccoli, apples, and tea with CVD (58).

This is but a small sample of epidemiological studies that have been undertaken to gauge the relationship between the consumption of apples or

their phytochemicals and risk of chronic disease. As can be seen from some of these studies, apples and apple phytochemicals can effectively prevent the incidences of some types of cancer, but not of cardiovascular disease, at least in this study. However, given the overwhelming amount of epidemiological evidence, showing the health benefits of apple consumption suggests that there is at least some protective effect of apples when it comes to cancer, one of the leading causes of death in the United States and around the world. Other diseases against which showed that apples have a protective effects are asthma, chronic obstructive pulmonary disorder when compared to other fruits and vegetables that have a high polyphenol content. In addition, apple consumption was also associated with improved lung function (56). Therefore the overwhelming amount of evidence suggests that apple phytochemicals have a protective effect with regard to at least some chronic. While thus far, the strongest association appears to be between colon cancer and the consumption of apples, the association between apple consumption or flavonoids derived from apples is currently of great interest and being investigated. While the mechanisms of some of these have recently started to come to light, further research is most certainly needed to elucidate the relationship between apples and other types of cancers and diseases against which apples may have a protective effect, and the mechanisms underlying these effects.

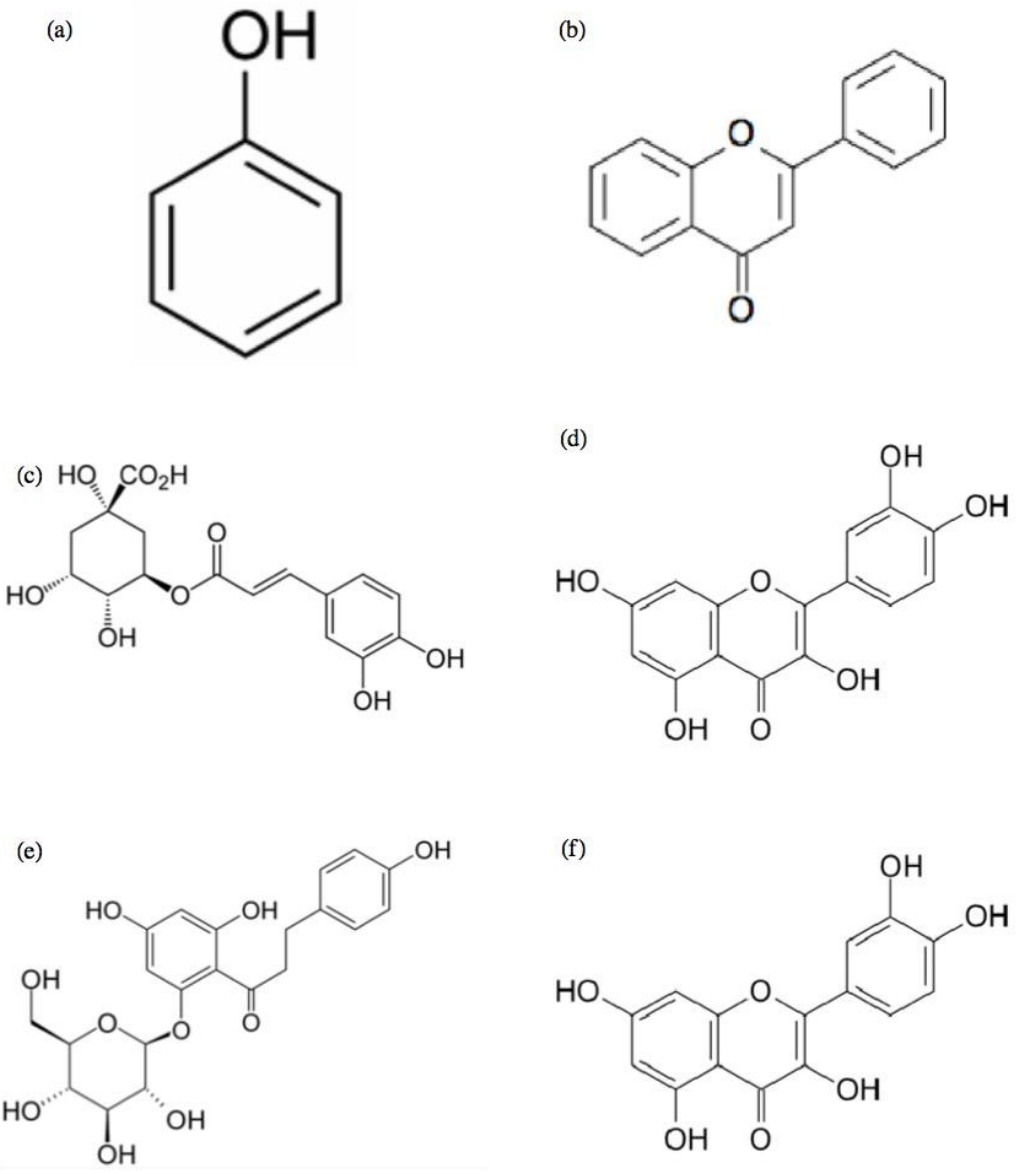


Figure 1.2 Major phytochemicals found in apples. Groups, classes and single components of apples: (a) Phenolic (b) Flavonoid (c) Chlorogenic acid (d) Catechin (e) EGCG (f) Catechin

1.8 Definition and theories of aging

Aging is a nearly universal biological phenomenon that is influenced by a variety of endogenous and exogenous factors, which are governed by an organism's genetic predisposition, its environmental setting, and stochasticity. In its broadest sense, it can be defined as a progressive deterioration of virtually every bodily function over time, resulting in an increasing susceptibility to environmental challenge and a growing risk of disease and death (59). In recent years, a great deal of progress has been made in identifying the underlying mechanisms and environmental conditions that influence aging, however many unanswered questions still remain. Although different species age at different rates, several recent studies have shown that lifespan may be controlled by a small number of conserved pathways and pharmacological manipulation of natural products and drugs (60, 61). The correlation between aging and incidences of chronic diseases has been well established in the literature (6).

Many theories exist to explain the physiological deterioration that occurs in an organism over time. Among evolutionary theories of aging, the "disposable soma theory" and the "antagonistic pleiotropy" theory are the most regarded. Likewise, the classical "free radical theory of aging" and more recently the "green theory of aging" provide alternative explanations for how organisms accumulate various types of damage that can lead to aging.

1.8.1 Free radical theory of aging

According to the Free Radical Theory of Aging, aging is a result of the accumulation of molecular damage caused by free radicals, highly reactive molecules that contain one or more unpaired electrons (62). Reactive oxygen

species (ROS), which are the most prevalent types of free radical oxidants, include superoxide anions, hydroxyl radicals, hydrogen peroxide, and peroxyl radical, and have been implicated in a number of degenerative and chronic diseases including cancer (63). In 1972, Harman refined the theory, suggesting that superoxide anions produced in the mitochondria are the major agents of damage that lead to the aging phenotype (64).

Free radicals emanate from both internal and external sources. Major endogenous sources of free radicals include the mitochondrial electron transport chain, peroxisomal fatty acid synthesis, metabolism of xenobiotics via oxidation/reduction of cytochrome P450, reaction with nitric oxide, as well as inflammation associated with pathogenesis (63). External sources include air pollution, ionizing radiation, and exposure to chemicals. Under normal physiological conditions, a cell's internal environment is maintained in a state of homeostasis in which there is a balance between oxidants and antioxidants, substances that scavenge free radicals. When the production and leakage of oxidants overwhelms the amount and scavenging capacity of antioxidants, oxidative imbalance occurs, resulting in molecular damage to DNA, proteins and lipids. While the Free Radical Theory of Aging accurately predicts the damage and deterioration that is caused by free radicals over time, evidence thus far only exists for a correlation between the accumulation of this damage and ROS production and no direct link to causation of aging has ever been established (65, 66). This has caused some to question its validity as a viable explanation for the aging process. In particular, several groups have recently published reports that contradict some of the theories predictions. For example, the deletion of some or all of the superoxide dismutases present in *C. elegans*, appears to have no effect on lifespan, yet still decreases oxidative

damage (67, 68). Some groups have suggested that it may be time to rethink the theory's tenets by building rather than setting aside its predictions. One such theory is the "green theory of aging."

1.8.2 Green theory of aging

According to the Green Theory of Aging, toxic byproducts of metabolism accumulate within the cell and lead to aging. These byproducts arise as a result of initiating events, including (but not limited to) ROS, UV radiation, and a variety of stochastic perturbations that lead to the formation of a wide range of secondary deleterious and toxic metabolites and cause biochemical damage within the cell. Examples of byproducts that are generated and broken up within the cell are lipofuscin, Advanced Glycation End-products (AGEs) and byproducts of oxidation such as lipid peroxidation debris and cross-linked proteins. Likewise, substances that enter the organism from outside, such as those found in food, drugs (in humans), and chemicals found in the environment, need to be metabolized in order to be eliminated. The system of enzymes that is responsible for performing this metabolism consists

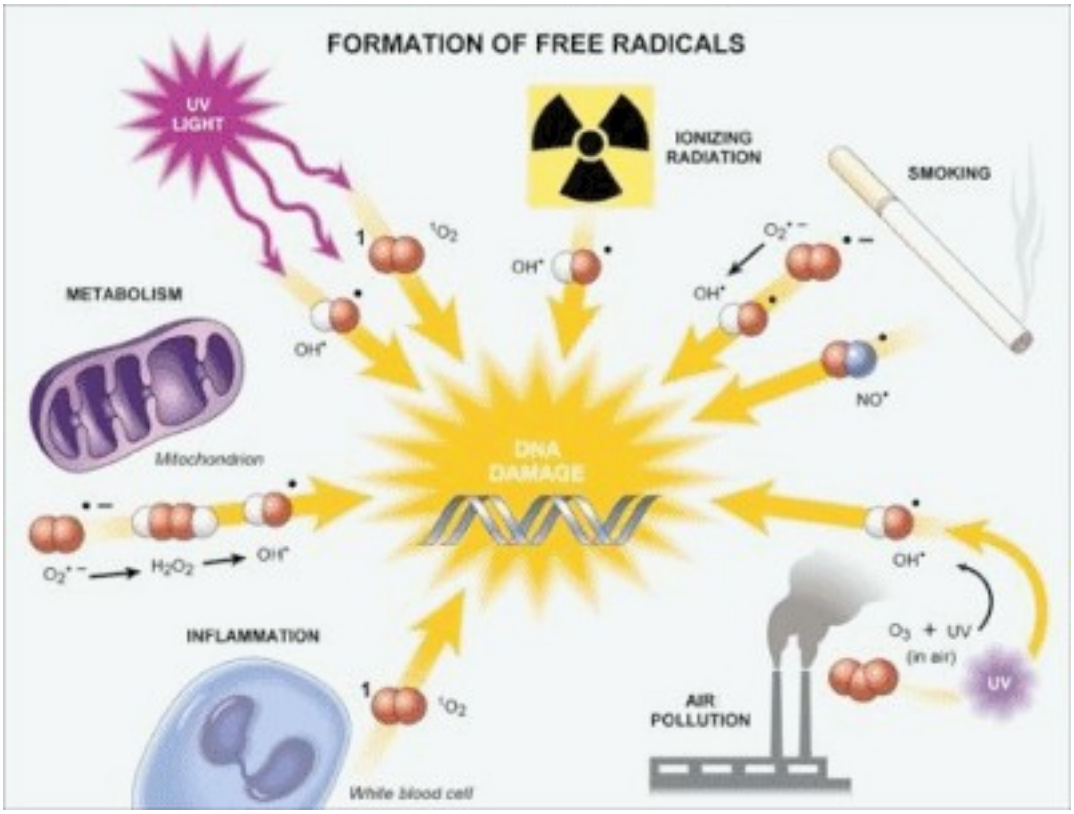


Figure 1.3 Formation of free radicals (adapted from Liu, RH presentation)

of Phase I enzymes, in particular those in the P450 family, and Phase II enzymes such as glutathione. Phase I enzymes add functional groups to make the chemical bioactive, usually by hydroxylation, and to prepare it to be conjugated by Phase II enzymes, which make them insoluble and allow their removal from the body. Thus, metabolism and excretion of toxic endobiotic and xenobiotic metabolites is the key to keeping the cellular environment junk free and to promoting longevity. However, the process of constantly “cleaning out” molecular junk is energetically costly, therefore a cell must carefully budget its resources and sometimes cannot carry the cleaning out even when it is necessary. As an organism ages, it accumulates more and more molecular junk, which becomes increasingly energetically costly to remove. If left inside the cell, this junk accumulates until it starts reacting within and causing deleterious damage to the cell. However, fitness may be optimized by investing only the minimum resources required into somatic maintenance processes, to increase the chances of survival through reproduction (69).

There are several lines of evidence to support this theory. McElwee *et al.* have shown that in microarrays studies of IIS and dauer nematodes, there is an enrichment of gene classes that code for cytochrome P450, short-chain dehydrogenase/reductase, UDP- glucuronosyl transferase, and glutathione S-transferase (70). The genes are mainly involved in the detoxification of toxic molecular rubbish. Similarly, according to Ayyadevara *et al.* a subset of glutathione-S-transferases (GSTs), which are Phase II enzymes, is overexpressed in the long-lived IIS mutant. In addition, life extension is fully reversed upon deletion of CeGSTP2-2 (a particular type of GST) expression (71). The green theory of aging is complementary to the disposable soma theory discussed below.

1.8.3 Disposable soma theory of aging

According to the disposable soma theory of aging, an organism must allocate resources between maintenance of somatic tissues and reproduction, which results in a trade-off between longevity and reproductive fitness (72). Interestingly, longevity assurance genes, of which detoxification genes are a major subset, are also energetically costly because they are required for the maintenance of the soma. In the wild, an organism is faced with a finite food supply, and must decide whether to expend resources for food energy and metabolism, or to save them for reproduction. If more resources are spent on reproduction at the expense of maintaining one's own soma, the organism would shorten its own lifespan. In contrast, if the organism allocates fewer resources to reproduction and spend more energy on its own maintenance, it will increase its chances of survival. While it makes sense from an evolutionary perspective for organisms that must survive in the wild, this theory has been called into question by a number of laboratory studies in *C. elegans*, which have shown that organisms can live longer with no effect on reproduction, and that reproductive capacity is reduced without affecting lifespan (73). Interestingly, other studies in *C. elegans* have shown that signaling from the germline and somatic gonad can have an effect on lifespan, without affecting reproductive capacity.

1.8.4 Antagonistic pleiotropy theory of aging

According to the theory of antagonistic pleiotropy, genes that are beneficial early in life may have detrimental effects later in life and thus led to aging. In particular, those genes that are selected for before reproduction can have negative consequences when the organism is no longer reproductively

viable because from an evolutionary standpoint their original function has been fulfilled. Therefore, ironically, they would promote lifespan early in life while shortening it late in life (74). Several examples of such genes exist in the literature. One is tumor suppressor p53, which confers protection against cancer (and death) by preventing abnormal cells from continuing to proliferate. However, this ability to stop cell proliferation is deleterious if it interferes with the proliferation of normal cells, such as stem cells for example, which are needed by the organism for tissue renewal as it ages. A recent review by Ungewitter and Scrable suggests that at least one other gene that is evolving independently, SIRT1 is required in for p53 to exert its deleterious effects late in life (75). Another example is the hormone testosterone in human males. Higher levels of testosterone lead to increased fertility early in life while causing decreased fitness later in life because of a higher risk of prostate cancer early in life lead to increased fertility (76).

Many other aging theories exist (59). However, evolutionary biology coupled with studies from model organisms and those in the wild, provides a useful framework against which to view such theories. Importantly, the role of molecular biology and model organisms in aging research is important in refining the major theories and enhancing our ability to understand the mysteries of the aging process.

1.9 *C. elegans* as an aging model

C. elegans is a popular model system in aging research for a variety of reasons. Under standard laboratory conditions, the organism has a short generation time of approximately 14-21 days and is easy to grow and culture in the laboratory at temperatures ranging from 16ºC to 27ºC. Molecular

genetics methods have been well characterized and tested, and more than 70 gerontogenes have been identified (77). Some, such as insulin signaling (see below), play a key role in mammalian regulation of age-related diseases. Additionally, worms and mammals share several common aging features. First, oxidative stress and the accumulation of reactive oxygen species and their byproducts have been shown to correlate with or contribute to aging and decrease lifespan in both humans and worms (78, 79). Second, calorie restriction, the only intervention that extends lifespan in mammals, extends the lifespan in *C. elegans* (80). Third, sarcopenia, the wasting away of muscle tissue is associated with the aging phenotype in both humans and worms (81). Additionally, as they age, both humans and worms undergo reproductive senescence and become infertile. The amount of information and informatic tools available for the molecular analysis of *C. elegans* is arguably the best of any model organism. In particular, the development of RNAi technology in *C. elegans* (for which Fire and Mello received the Nobel prize in 2006) to knock out genes and study their function has been an invaluable tool in aging research.

Despite these useful features, some disadvantages also limit the worm's utility as an aging model (77). The worm's small size may prevent detailed biochemical and pathological study in some cases. Physiologically, the worm also lacks certain critical systems such as the immune system. Evolutionarily, *C. elegans* is quite far removed from mammals (hundreds of millions of years), which means that many critical human genes have no homologs and vice versa. Additionally, the worm is covered with a tough cuticle that is difficult to penetrate, which may make it a challenge to study drug response in certain cases. Despite these shortcomings, a substantial

body of scientific literature attests to the fact that *C. elegans* is an excellent model system to study aging (82, 83).

1.9.1 Aging mutants in *C. elegans*

In *C. elegans*, a number of longevity pathways which shed light on the aging process been identified. The elucidation of these pathways has been facilitated by the production of long and short-lived mutants. Below is a summary of the major mutant categories and their effects on lifespan.

1.9.2 Insulin signaling pathway: role of DAF-16 in lifespan extension

In *C. elegans*, the best-studied longevity pathway is the *daf-2*/IGF/insulin signaling pathway also known as the IIS pathway. This pathway regulates dauer formation (a diapause state which the nematode enters in times of stress), development, stress resistance and lifespan. Mutation of *daf-2*, the insulin-like receptor, extends *C. elegans* lifespan by up more than 2-fold (84). Interestingly, the lifespan extension of IIS mutants depends upon only a single transcription factor, *daf-16*, which regulates hundreds of downstream targets. *daf-2* mutants are more resistant to many different types of stress, including oxidative, heat and UV stress and have improved fat storage and metabolism. DAF-16 is negatively regulated by DAF-2, a tyrosine kinase receptor that is the *C. elegans* insulin/IGF receptor ortholog. Upon binding of an insulin-like ligand, DAF-2 signals AGE-1, a phosphoinositol-3-kinase (PI3K), which in turn activates PDK-1 and AKT-1/AKT-2. When activated, AKT-1 and AKT-2 phosphorylate DAF-16, sequestering it from the nucleus. Therefore, only a very low amount of DAF-16 is normally present in the nucleus under normal, non-stressed conditions. However, in times of stress

(e.g. starvation, heat or overcrowding) DAF-16 is dephosphorylated and translocates into the nucleus. Here, it can bind to the promoter sequences of many genes that regulate stress response, including oxidative and heat shock, metabolism, fat storage and lifespan. Importantly, these genes can be both up and down regulated in response to DAF-16 binding. For example, the genes that code for the antioxidant enzymes SOD-3 and CTL-2 are upregulated, whereas yolk protein genes VIT-2 and VIT-5, as well as PEP-2, an oligopeptide transporter are downregulated (86).

Studies on tissue specificity have revealed insulin signaling to be tissue specific. Wolkow *et al.* have shown that *daf-2* or *age-1* signaling from the neurons is sufficient to affect lifespan (87). In addition, Libina and colleagues have shown that overexpression of *daf-16* in the intestine (where fat storage and metabolism take place), is sufficient to increase lifespan (88). Importantly, the insulin signaling pathway and its effects are conserved across species, including flies and mice.

1.9.3 SIR2.1

Silent information regulator (SIR) proteins (also known as sirtuins), of which *sir2.1* is a member, belong to a family of NAD+-dependent deacetylases that play an important role in gene silencing, DNA repair and aging in a diverse group of organisms (89). Sirtuins exert their major effects in the cell by removing acetyl groups from specific target proteins. This deacetylase function depends on the intracellular concentration of nicotinamide adenine dinucleotide (NAD), a molecule involved in metabolism. This molecule can exist in two states, oxidized and reduced. The oxidized form is the one that greatly enhances *sir2.1* activity. In *C. elegans,* increased expression of *sir2.1*

has been shown to extend lifespan (90), and *sir2.1* has been shown to both interact with and have distinct functions from *daf-16,* and has been proposed to be partly responsible for the increase in lifespan under caloric restriction (91). Small molecules that affect *sir2.1's* enzymatic activity have been identified. Among these is resveratrol, a phytochemical that is found in a variety of plants. It has been shown that resveratrol extends lifespan in *C. elegans* in a manner that is dependent on *sir2.1* but independent of *daf-16* (92).

1.9.4 Oxidative stress mutants

In *C. elegans*, oxidative stress and the free radical theory of aging (discussed in the previous section) have been a subject of great study and much contention. While initial evidence seemed to indicate that oxidative stress and aging may have a causal link (93-95), several recent studies have called this hypothesis into question (67).

In *C. elegans*, the use of mitochondrial mutants, the most popular of which are *mev-1, gas-1*, *isp-1* and *clk-1* has been used to test for effects of various interventions on oxidative stress. In addition, the use of free radical generating chemicals such as paraquat, juglone and hydrogen peroxide has also been popular. In the following section, an overview of the major OS mutants will be presented followed by a review of several recent reports which seem to suggest that there is no cause and effect relationship between OS and aging.

mev-1 animals have a missense mutation in the *cyt-1* gene coding for the succinate dehydrogenase cytochrome *b* large subunit of complex II. The animals in this strain are hypersensitive to oxygen, produce excessive

amounts of superoxide anions in their mitochondria and have a shortened lifespan. In addition, they also have lower superoxide dismutase levels, reduced glutathione levels, and higher lactate concentrations (96).

gas-1 mutants have a mutation in a subunit of mitochondrial complex I that is required for oxidative phosphorylation. Because of this, they have a strongly reduced complex I dependent metabolism and an increased metabolism of complex II. The mitochondria of these animals appear normal. *isp-1* is an iron sulphur protein that is a subunit of the mitochondrial complex III in the mitochondrial membrane. Complex III catalyzes electron transport from ubiquinol to cytochrome c.

The isp-1 mutants show reduced oxygen consumption and decreased sensitivity to ROS. These mutants are long lived. Because the ISP-1 mutation affects the rate of development and production, the *isp-1;ctb-1* double mutant is often used to ensure that the slow embryonic development is not a confounding factor. The CTB-1 gene encodes the cytochrome b protein of mitochondrial complex III. As stated above, it rescues the abnormal development of *isp-1* mutants and also increases their resistance to paraquat.

Another gene that is involved with regulating mitochondrial respiration in *C. elegans* is CLK-1. This gene is required for biosynthesis of Coenzyme Q (Ubiquinone), which reduces the amount of superoxide anion in the mitochondrial respiratory chain. CLK-1 codes for a highly conserved demethoxyubiquinone (DMQ) hydroxylase that is necessary for normal physiological rates of growth, development, behavior, and aging, and normal brood sizes (80). *clk-1* mutants have an increased resistance to oxidative stress. The metabolic activity and ATP levels of *clk-1* mutants are unchanged. *Clk-1* mutants have an extended lifespan.

1.9.4.1 Knockout models

Besides the use of mutant and/or double or triple mutant animals, several strategies have been employed to examine the relationship of oxidative stress and aging in *C. elegans*. The most popular of these has been to knock out the antioxidant enzymes that are responsible for the endogenous oxidative stress response. The idea behind these experiments is that deleting some or all of these enzymes will increase oxidative stress. If the free radical theory of aging is correct, this increase of oxidative stress should be followed by a decrease in lifespan. Interestingly, it was recently shown that this is not the case. Two groups systematically knocked out every one of five superoxide dismutases in the worm and showed that there was either no change, or sometimes even an increase in lifespan. This suggests that in *C. elegans*, oxidative stress is not causal for aging. However, damage from ROS in conjunction with damage from other sources still likely contributes to aging (66,67). Moreover, a study published by Schulz *et al.* has shown that oxidative stress can in some cases actually increase lifespan. These researchers showed that when *C. elegans* were put on a glucose-restricted diet, they had increased mitochondrial respiration and higher production of ROS, yet still lived longer than the controls. They hypothesized that the observed increase in lifespan was due to hormesis within the mitochondria, which they termed “mitohormesis.” Interestingly, when antioxidant treatments were applied to animals on a glucose-restricted diet, lifespan actually decreased (97). This seems to counter one of the fundamental tenets of the free radical theory of aging, that ROS causes damage and decreases lifespan, and antioxidants rescue this effect.

It is important to note that while at present there is much contention regarding the validity of the free radical theory, it still provides a powerful explanation for aging-related states, which implicate ROS damage as the main culprit. Alzheimer's disease is one such example. Additionally, the free radical theory of aging has been incorporated into other, new theories such as the green theory of aging (69).

1.10 Calorie restriction and other mutants

Calorie restriction (CR) is the only known intervention that has been shown to increase lifespan in organisms ranging from yeast to primates. In *C. elegans*, CR can be achieved on one of three ways: dilution of the bacterial source, culture in semi-defined liquid medium in the absence of *E. coli* or through the use of mutants. The first reported case of calorie restriction in *C. elegans* was done by diluting the concentration of *E. coli* is S medium. Upon treatment, worm lifespan increased and fertility decreased (98). Similarly, Hosono *et al.* decreased food intake by reducing the thickness of the bacterial lawn on solid agar plates. This was accomplished by reducing the nutrient content of these plates (99).

The third way by which calorie restriction of *C. elegans* can be achieved is through the use of mutants. The major example of this is the *eat-2* mutant strain, which has a pump rate that is naturally lower than wild-type. This strain has a mutation in the ligand-gated ion channel subunit of the pharynx, which causes the worm to pump at a much slower rate than normal and therefore take in less food per unit time. The EAT-2 gene is required for normal life span and defecation. As expected, animals that have a mutation in *eat-2* are longer lived compared to wild-type.

Another pathway, which is implicated in extending lifespan via CR is TOR (target of rapamycin). Mutants of this pathway have been suggested to modulate life extension by CR (100). TOR is a conserved protein kinase that plays an essential role in regulating growth and metabolism in response to nutrients and growth factors. TOR interacts with the regulatory associated protein of TOR (raptor) to allow transduction of nutrient signals to a wide range of downstream cellular processes, such as mRNA translation, ribosome synthesis, expression of metabolism-related genes and autophagy. One of the major functions of TOR is to promote protein synthesis by activating S6K and inhibiting eukaryotic translation initiation factor 4E-binding protein (4E-BP). Modulation of mRNA translation plays an important role in lifespan determination in many species. In *C. elegans*, mutations in the TOR ortholog *let-363* leads to developmental arrest at dauer (third larval stage) and intestinal atrophy. The arrest at the third larval stage is also observed with homozygous but not heterozygous mutants of *daf-15*, the *C. elegans* ortholog of raptor. Heterozyous mutants of raptor have an increased lifespan. Additionally, inactivation of *rsks-1*, the *C. elegans* ortholog of S6K extends lifespan. S6K is phosphorylated by Akt/PKB, suggesting that there is crosstalk between the IIS and TOR pathways (101). RNAi of *let-353* extends lifespan of wild-type animals, but not of *pep-2* mutant animals. PEP-2 is a proton-dependent carrier, which regulates the uptake of di- and tripeptides. Evidence suggests that *pep-2* acts upstream of TOR through PI3K and AKT (100). Major questions about the role of TOR in CR-mediated life extension still remain. It is still uncertain for example whether inhibiting *let-363* by RNAi further extends lifespan of the *eat-2* mutant (102).

1.11 Pharmacological manipulation of aging

C. elegans is a good model for aging research because as they grow older, worms experience a variety of behavioral and physiological declines similar to higher mammals, including humans (77, 98). As they age, worms move more slowly and exhibit sarcopenia - the progressive deterioration of muscle tissue. They also become infertile, and accumulate oxidized proteins and lipofuscin, both of which are hallmarks of aging in most species (77, 98). At the gene and protein levels, *C. elegans* share a number of homologs with human genes and a conserved protein network involved in aging (103, 104). Besides being a biologically relevant aging model, worms are easy to culture and have a short life cycle, allowing for rapid replication of experimental treatments.

In *C. elegans*, standard assay conditions to study the pharmacology of drugs and interactions with genes have been described (105). To date, a variety of human pharmacological interventions ranging from vitamins and individual phytochemicals to clinical drugs and antioxidant supplements are known to extend lifespan or delay physiological aging in *C. elegans* (61, 106). Recently, whole-plant phytochemical extracts have also been tested and found to have a significant life-extending effect (107-109). Some of these studies are reviewed below. However, before proceeding, I feel that it is important to point out some caveats that make the interpretation of this information less than ideal.

While pharmacological manipulation of aging is an exciting and important area of research because it has the potential to alleviate age-related pathologies, a number of factors must be noted when analyzing data and trying to compare different studies. First, background of the control strain such

as N2 vs. the sterile *fem-1* must be noted when comparing studies. A number of reports have been published using same or similar compounds, yet different control strains making it harder to draw direct comparisons (94, 110, 111). Second, temperature at which the experiment is performed plays a major role. Since temperature has a direct effect on lifespan in *C. elegans*, there usually exists an optimal temperature at which the maximum effect of a pharmacological treatment is observed. Thus if an experiment is only done at one temperature and no effect is observed, this result may be due to pleiotropy, or masking of the effect(s) between temperature and compound(s) of interest. However, a number of groups have taken this into account and performed pharmacological lifespan analyses under a range of different temperatures to show that the effects are robust and reproducible at a range of temperatures. Thirdly, the *E. coli* bacteria source plays a vital role. Usually, the uracil-requiring OP50 strain is used as the food source. Because bacteria can metabolize drugs, it is important to ensure that the effect is not due to the bacteria, but rather due to the drug acting directly on the worm. This caveat can be overcome by killing the bacteria. A variety of methods exist to do this, the most popular of which are UV irradiation and addition of antibiotic to which the bacteria is sensitive. However, most reports that have been published to date have only reported results on live bacteria. Thus, the possibility exists that the effects are due at least in part to bacterial metabolism rather than direct drug action on the worms. Fourthly, some compounds only exert their effects when administered at hatching, while others need to be administered at sexual maturity to avoid deleterious developmental effects. Many studies only publish one or the other, but not both. Sometimes it is difficult to tell at what stage the treatment began altogether. Fifth, it is important to know if the

compound affects the number of progeny of the animals. Many compounds that extend lifespan show reproductive toxicity, reduced metabolism and reduced respiration. Sixth, it is possible that the compound may be toxic without exerting a phenotypic effect by inducing caloric restriction. Traditionally, the pump rate is measured to ascertain whether worms are feeding normally. Seventh, a lot of compounds must be dissolved in a vehicle solvent that is not evaporated off by the end of its preparation. Therefore, the appropriate controls must include the solvent at the same percentages as the treatment groups. Moreover, lifespan assays are usually done on NGM petri plates and the pharmacological extract of interest must be dissolved at no less than 55°C (to ensure that the agar does not solidify), along with the NGM media, this can raise issues of stability of the pharmacological agent as this high of a temperature may disrupt its structure. Eighth, some compounds such as Vitamin E are light sensitive and degrade quickly upon contact with light. Four of the papers that are reviewed below used this vitamin to show an affect on lifespan (or lack thereof). However, not a single paper reported whether they covered the experimental petri dishes. This may mean that none of them did, but given that it is a well-known fact that Vitamin E is unstable, it would be useful to know the details of the experimental setup. Perhaps the lack of this information may also explain some of the observed differences between these researchers. If they plates had been covered, careful attention must be paid so as not to induce hypoxia to which *C. elegans* are extremely sensitive. Finally, a nice review by Gems and Partridge summarizes a list of important caveats to keep in mind when doing experiments using mutant strains to test for drug interactions (112).

To date, close to 30 reports on the pharmacological manipulation of

aging in *C. elegans* have been published. More often than not, at least some of these caveats are not addressed in these reports. Lack of standardization may make it difficult to compare studies and halt the pace of progress. These caveats not withstanding, *C. elegans* remains one of the best models to study aging. For the ease of this review, studies will be grouped into four categories: vitamins, antioxidants, drugs and natural products.

1.11.1 Effect of vitamins

In 1983, Zuckerman and Geist published the first report of an intervention to extend lifespan in *C. elegans* (113). These researchers showed that Vitamin E (d,1-α-tocopherol) at a dose of 200 μg/mL can increase both the mean and the maximum lifespan of *C. elegans* if treatment is started during the prereproductive period. In a subsequent publication, Harrington and Harley confirmed these findings and also found that Viatmin C (L-ascorbic acid) or a Vitamin E (d,1-α-tocopherol) and C combination had no effect on lifespan. In addition, these researchers found that Vitamin E decreased fecundity and increased the mean day of reproduction (110). Vitamin E consists of two major forms, the tocopherols (α-tocopherol, β-tocopherol, δ-tocopherol and γ-tocopherol), and the tocotrienols (α-tocotrienol, β-tocotrienol, δ-tocotrienol and γ-tocotrienol). The tocopherols, in particular α-tocopherol is the predominant form absorbed by the human body and found in tissues of most animals. The major difference between these two forms is three double bonds (e.g. lack of saturation of the isoprenoid tail) found in the tail of tocotrienols. However, the antioxidant activity of these two forms varies greatly. Although the tocotrienols only have about 1/3 the biological activity of tocopherols, they have 40-60% more antioxidant activity. Adachi and Ishii

found that a tocotrienol fraction, but not a tocopherol fraction increased the mean lifespan in *C. elegans*. This increase was paralleled by a lowering of protein carbonylation and an improved survival upon exposure to UVB irradiation, which induces cumulative oxidative stress by generating hydrogen peroxide and hydroxyl radicals. Interestingly, the survival was improved when the tocotrienol fraction was applied both pre and post – UVB treatment. Surprisingly, this report differed from the two previous findings that α-tocopherol increased lifespan in *C. elegans*. There may be several explanations for this. First, the researchers administered a much lower dose than previously reported (highest dose used was 80 μg/mL instead of 200 μg/mL) since they wanted to use the same dose for both forms of vitamin E and they had reason to believe that the 80 μg/mL dose would be more efficient for the tocotrienol fraction. Second, they used a tocopherol fraction rather than using the α-tocopherol isomer, as did previous reports. Although α-tocopherol is the most biologically active form, it may be that combining all four tocopherol isomers abrogates α-tocopherol's activity when assessing for lifespan increase and protein oxidation. Thirdly, it was not clear from this study at what stage of development treatment with Vitamin E was begun. The two previous studies, which reported an effect started treating at hatching, it was not immediately clear from the information supplied in the paper whether this was also the case here (94). Another study by Zou *et al.* found that Vitamin E extends lifespan in *C. elegans* even when supplied at young adulthood (111). These researchers found that contrary to the two early studies, α-tocopherol at the high dose of 200 μg/mL slightly reduced mean lifespan compared to the control. In contrast, γ-tocopherol increased mean lifespan at both 20 and 200 μg/mL. Neither dose affected maximum lifespan, and mixing both forms in

equivalent ratios produced no significant effect on lifespan. The researchers concluded that α-tocopherol supplementation could suppress any benefits of γ-tocopherol supplementation in this model. A possible explanation as to why these results differed from the two early studies could be that treatment in this case was begun post-reproductively. Also, the sterile *fem-1* strain was used instead of the wild-type N2. Interestingly, in this study a comparative approach was used to test the effects of α- and γ- tocopherol on two fly species, *D. melanogaster and A. ludens*. In *D. melanogaster,* supplementation had no effect on males and slightly reduced the mean lifespan of females. In *A. ludens*, there was no effect on both males and females (111).

1.11.2 Effect of Drugs

The anti-aging effects of several clinical drugs have been tested in *C. elegans.* Evanson *et al.* screened 19 FDA approved anticonvulsant drugs and found that ethosuximide extended mean lifespan by 17%. In a follow-up study of the structure-activity relationship, a group of structurally-related anticonvulsants was tested. Of these, it was found that trimethadione increased mean lifespan by 47% and DEABL by 31%. These drugs modulate neural activity in vertebrates and were found to regulate neuromuscular activity in worms. The authors concluded that the anticonvulsant activity of these drugs is directly responsible for extending lifespan and therefore that neural activity plays a role in the regulation of aging. Two models were proposed for how this may occur. In the first model, neurons promote lifespan directly by modulating processes that are essential for life. In this model, age-related decline is delayed by anticonvulsant drugs, which delay neuronal degeneration. Alternatively, activity of the neurons contributes to aging by

accelerating aging of nonneuronal cells, which promote survival. Here, anticonvulsant drugs may function to decrease the levels of neuronal activity. The authors concluded that, the second model is better supported by the evidence. Specifically, in nematodes treated with these drugs, egg laying was increased because eggs were deposited earlier, there was increased motility (fast body movement) and hypersensitivity to the acetylcholinestarase inhibitor aldicarb was observed. Additionally, the IGF pathway was shown not to be required for the actions of the drug. The authors concluded that because drugs that affect neural activity delay the aging of nonneronal reproductive tissues, model 2 was probably the most likely explanation (114,115).

Similarly, Voisine *et al.* performed a screen of 16 drugs used to treat Huntington's disease and found that mithramycin and lithium chloride, extend lifespan of the *pqe-1* mutant in a *daf-16* independent manner. The *pqe-1* mutant is a model of Huntington's disease in which polyQ mediated neuronal degeneration and aging are accelerated. By assaying neuronal survival using GFP fluorescence and a dye, the researchers showed that these drugs slowed the degeneration of neuronal cells (116). Last, but not least, Petraschek and colleagues performed a screen of 80,000 compounds and identified a drug mianserin used to treat depression in humans that extends maximum lifespan by up to 31%. A detailed description of the experiments performed and mutants tested is beyond the scope of this review, however, a variety of mutants were used to determine that mianserin affects serotonin synthesis, serotonin re-uptake at synapses, or either of two G-protein-coupled receptors, data which were in agreement with data on humans (117). This study combined with the two reviewed above strongly suggest that neuronal activity and aging are closely linked.

1.11.3 Effect of Antioxidants

Several studies have examined the effects of administering antioxidants to *C. elegans* as a way to extend lifespan. According to the free radical theory of aging (reviewed above), free radicals are the main agents of damage to DNA, proteins and lipids. These radicals produce byproducts that accumulate in the cell over time and lead to cellular senescence and aging. Melov *et al.* have reported using superoxide dismutase and catalase mimetics, EUK-8 and EUK-134, respectively, to extend lifespan of *C. elegans*. These researchers found that in liquid culture mean lifespan increased by up to 44%. To address the mechanism of action, the researchers showed that the drugs extended the lifespan of the *mev-1* mutant, a strain with a mutation in the complex II of the mitochondrial respiratory chain that is defective in reactive oxygen species (ROS) metabolism (reviewed above). It was also found that these drugs did not significantly affect growth or fertility (93). In agreement with this result, Sampayo and colleagues used the same compounds and found that there was a dose-dependent increase in survival of paraquat treated and heat shocked animals, further providing proof that these compounds act as antioxdants *in vivo*. In addition, these researchers showed that this effect is independent of insulin signaling (106). However, in a follow-up study, Gems and colleagues found that the increase in lifespan was not reproducible (and showed that it even decreased) calling into question the results of the first study as well as the free radical theory of aging. The researchers did however find that drug treated animals had elevated levels of superoxide dismutase activity. Moreover, they also found that treatment with the drug mimetics improved survival animals exposed to oxidative stress, but not those

maintained under normal conditions. The authors concluded that antioxidant mimetics do not increase lifespan under normal conditions and speculated that the discrepancy in the results compared with the previous group was due to different culturing conditions (the first group used liquid culture that the authors suggested may have generated additional oxidative stress). These studies show that for the reasons discussed above, culturing conditions are crucial when interpreting lifespan studies (118,119).

Resistance to stress has been linked to increased longevity (120). A recent study by Benedetti *et al.* tested a wide range of antioxidant compounds by utilizing a screen for thermotolerance (121). Candidates that were found to improve thermotolerance were further tested for their lifespan effects. Out of a total of six compounds, lipoic acid, propyl gallate, trolox and taxifolin were found to extend lifespan under normal culturing conditions. Interestingly, while there were no deleterious effects on fecundity, the authors found a delay in fertility for lipoic acid and a reduction in the size of the progeny of lipoic acid and propyl gallate treated animals. To address the mechanism of action, the authors used several GFP reporter strains: *sod-3, gst-4, hsp16.2* and *cyp-35A3*. CYP-35A3::GFP showed a 2-fold increase in expression upon treatment with trolox. This gene is regulates the xenobiotic stress response. The authors concluded that the xenobiotic stress response may contribute to the observed lifespan extension. Alternatively, since heat shocked worms show dramatically increased production of ROS it was suggested that these compounds can directly detoxify ROS. Perhaps, death from thermal stress may be mediated by oxidative stress.

Several other studies looked at the use of ions and nanoparticles as antioxidants on *C. elegans* lifespan. Kim *et al*. showed that platinum

nanoparticles increased the lifespan of N2 worms, increase survival upon paraquat treatment, attenuated ROS by increasing the lifespan of *mev-1* mutants, and decreased levels of lipofuscin (95). Lin and colleagues found that manganese ion supplementation enhanced resistance to oxidative stress, and rescued the lifespan of *mev-1* mutant animals. Interestingly, the authors also observed accelerated development of wild-type animals. The authors proposed that manganese may act as a free radical scavenger or alternatively may regulate other protective factors (122). Vitamin E and its various forms, which were reviewed above is also a potent antioxidant. Taken together the data in this section show that, while antioxidants may come in many different forms, they have a common activity – to alleviate the damage caused by ROS and increase lifespan in some, but not all cases.

1.11.4 Effect of Natural Products

Recently, natural products or compounds derived from them have been tested in the lifespan manipulation of *C. elegans*. These included both single compounds as well as plant extracts. The most familiar example from recent history is resveratrol, a stilbene found in grapes, peanuts and other plant foods. This phytochemical was shown to extend lifespan in *C. elegans* by anywhere between 10-52% (89, 123). This effect was mediated through the *sir2.1* pathway. For a time, this finding generated a lot of excitement as study after study in different model organisms found that this effect on lifespan or age-related decline was reproducible (124-126). However, another study showed that resveratrol's effects on the lifespan of both *C. elegans* and *D. melanogaster* were variable. Whereas in some cases it increased lifespan slightly, in others it did not, suggesting that the mechanism of action is more

complex than previously thought, and that it is at least in part dependent on culturing techniques (127). It is important to note that resveratrol does not act solely via *sir2.1.* In a recent article, Pirola reviewed the many molecular targets of this interesting compound, which include the phase II detoxification system and AMP kinase (128). Other single phytochemicals, which have been shown to extend lifespan, increase stress resistance, or both include kaempferol, rutin fistein, quercetin and EGCG. The two latter examples are discussed in greater detail in section 2.4.

Three whole plant extracts have been shown to increase lifespan in *C. elegans.* A number of studies using the *Ginko biloba* extract EGb 761 in *C. elegans* have been published. *Ginko biloba* has been used medicinally in China for thousands of years to enhance memory and prevent dementia. Luo *et al.* showed that amyloid beta aggregation was inhibited in an Alzheimer's disease model of *C. elegans* (129, 130). In this model, the human gene for $A\beta_{42}$ is overexpressed. The resulting amyloid beta aggregates lead to paralysis and death. Interestingly, only aggregates of a certain size cause this effect. Kampkotter *et al.* showed that EGb671 reduces stress sensitivity to ROS, expression of catalase and glutathione-S-transferase (131). Wu *et al.* have shown that the *Ginko biloba* extract EGb 761 increased the mean lifespan of wild-type worms by 8%. It was shown that oxidative stress was decreased in both the wild-type and *mev-1* mutant and that thermotolerance was increased following EGb 761 administration in both wild-type and *mev-1* animals (107). In another study with the same extract, Strayer *et al*. showed that *hsp16.2* expression was decreased following treatment. This effect correlated with the organism's increased survival upon thermal and oxidative stress. Without ruling out other possible effects, the authors concluded that reduced *hsp16.2*

expression led to reduced cellular stress (132). Cao *et al.* reported that EGb 761 decreased sarcopenia in a PD4251 model (which expresses GFP in body wall muscles and vulval wall nuclei) of *C. elegans*. The authors also observed an improvement in locomotion, body bending and pharyngeal pumping (133).

Plant adaptogen extracts have also been tested for their lifespan effects in *C. elegans*. Wiegant and colleagues showed that extracts of *Eleutherococcus senticosus* and *Rhodiola rosea* increased the mean lifespan of wild-type animals in a dose-dependent manner between 10-20%. In addition, both extracts also increased stress resistance against heat shock and oxidative stress and induced translocation of DAF-16 into the nucleus. The authors concluded that these adaptogens are experienced by *C. elegans* as mild stressors and thereby act to enhance general stress resistance and increase lifespan (109).

The only study to test the effects of whole extract fruit (berry fruits) phytochemicals on lifespan was done by Wilson *et al.* who showed that crude blueberry extracts increase lifespan and enhance thermotolerance of *C. elegans* (108). This study found that mean lifespan of *fem-1* worms was increased by 28%. Unlike in the *Ginko biloba* study, these authors did not find that whole plant extract increased lifespan by relieving oxidative stress (although they left open the possibility that low levels of oxidative stress may be quenched). The authors tested a variety of longevity mutants. The premise of these experiments was that blueberry treatment would not extend lifespan in mutant animals missing a gene required for blue berry's beneficial effect. Mutants of various known aging pathways, including *daf-16, sir2.1* and *eat-2* were tested. These pathways did not appear to be affected by the extracts. However, mutants of the OSR-1/UNC-43/SEK-1 osmotic stress resistance

pathway did not show increased lifespan. The authors thus concluded that this pathway was necessary for lifespan extension with blueberry extracts. To get at the fraction of blueberry extracts, that was responsible for the life-extending effect, the extracts were fractionated. It was found that the proanthocyanidin (PAC) fraction was responsible for the effect. This is to some extent surprising because these compounds are known to have low bioavailability, at least in humans. Nonetheless, this study was the first to consider the effects of food, and in particular berry fruits on the lifespan and healthspan of *C. elegans.*

CHAPTER 2

Apple extracts increase lifespan, healthspan and resistance to stress in *Caenorhabditis elegans*

2.1 Introduction

Natural products derived from plants represent a significant source of potential human therapeutic agents. Compounds found in these agents may aid in the maintenance of health and the prevention of chronic ailments such as cancer, cardiovascular disease, diabetes, Azheimer's disease, hypertension, and cataracts (4). Age is a major risk factor for developing many such chronic diseases. As an organism ages, it experiences a progressive deterioration of virtually every bodily function over time, resulting in an increasing susceptibility to environmental challenge and a growing risk of disease and death (59). Therefore, a key goal of aging research is to discover compounds that will aid in the prevention and treatment of age-related diseases, and delay the onset of aging and age-related morbidity.

The nonparasitic nematode *C. elegans* is a popular model in aging research because as they grow older, worms experience a variety of behavioral and physiological declines similar to higher mammals, including humans (77, 98). As they age, worms move more slowly and exhibit sarcopenia - the progressive deterioration of muscle tissue. They also become infertile, and accumulate oxidized proteins and lipofuscin, both of which are hallmarks of aging in most species (77, 98). At the gene and protein levels, *C. elegans* share a number of homologs with human genes and a conserved protein network involved in aging (103, 104). Besides being a biologically relevant aging model, worms are easy to culture and have a short life cycle, allowing for rapid replication of experimental treatments.

In *C. elegans*, standard assay conditions to study the pharmacology of drugs and interactions with genes have been described (105). To date, a variety of human pharmacological interventions ranging from vitamins and individual phytochemicals to clinical drugs and antioxidant supplements are known to extend lifespan or delay physiological aging in *C. elegans* (61). Recently, whole-plant phytochemical extracts have also been tested and found to have a significant life-extending effect (107-109).

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables is associated with a reduced risk of developing chronic diseases (1-3). Doll and Peto estimated that at least one-third of all cancers can be prevented by dietary modification (134). We and others have proposed that the health benefits of fruits and vegetables arise from interactions of complex mixtures of phytochemicals, the bioactive non-nutrient plant compounds that have been linked to reducing the risk of major chronic diseases. These compounds may possess additive and synergistic effects and provide benefits beyond those obtained from individual supplements (4).

Apples are so commonly consumed in the U.S. that they are the top contributor of fruit phenolics in American diets (38). Whole apple extracts had potent antioxidant effects and anti-proliferative activity against colon, liver and breast cancer cells *in vitro* in a dose-dependent manner (49, 50, 52, 135). They prevented DMBA-induced mammary cancer in rats, inhibited NFκB activation in human breast cancer MCF-7 cells, induced G1 arrest and decreased the expression of Cyclin D1 and Cd4 (11, 52, 54). Recent work at our lab also included the isolation of new, structurally diverse compounds from apple peels that had potent antioxidant effects and anti-proliferative activity

against MCF-7 breast cancer cells and HepG2 liver cancer cells (40, 51). Although many such molecular health effects of apple phytochemicals have been identified, little is known about their effects on aging. The objective of this study was to determine the effects of apple phytochemical extracts on lifespan, healthspan and stress resistance *in vivo* using *Caenorhabditis elegans* as a model for human aging.

2.2 Methods and materials

2.2.1 Chemicals and reagents

Acetone was purchased from Fischer Scientific (Pittsburgh, PA). Sodium carbonate was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ) and gallic acid from ICN Biomedical Inc. (Aurora, OH). Folin-Ciocalteu reagent, 5-fluoro-2-deoxyuridine (FUDR) and methyl viologen dichloride hydrate (paraquat) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.2 Extraction of apples

Fresh apples of the Red Delicious variety were purchased from Cornell Orchards (Cornell University, Ithaca, NY) and extracted using the method previously reported by our laboratory (50). Briefly, whole apples were sliced, blended in ice-chilled 80% acetone, homogenized and rotary evaporated under vacuum at 45°C until approximately 90% of the filtrate (and all acetone) had been evaporated. The concentrate was re-suspended in water, and no other solvent remained in the extract. Apple phytochemical extracts were frozen at –80^{o}C until use. The extracts have been characterized based on bioactivity-guided fractionation and structure identification using HR-MS, 1D and 2D NMR, and X-ray diffraction analysis using the method we reported

previously (40).

2.2.3 Determination of total phenolic content

Total phenolic content of apples was measured using a modified colorimetric Folin-Ciocalteu method (136). Briefly, volumes of 0.5 mL of deionized water and 0.125 mL of apple extracts diluted 1:10 in triplicate were added to test tubes. Folin-Ciocalteu reagent 0.125 mL) was added to the solution and allowed to react for 6 min. Next, 1.25 mL of 7% sodium carbonate solution was aliquoted into the test tubes, after which deionized water was added to adjust the mixture to 3 mL. The color was developed for 90 min, and the absorbance was read at 760 nm using a MRX II Dynex spectrophotometer (Dynex Technologies, Inc., Chantilly, VA). Absorbance values were compared to a standard curve of known gallic acid concentrations and expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh fruit ± SD for triplicate extract samples.

2.2.4 Strains, maintenance and culturing of nematodes

Strains used were: Bristol N2 (wild-type), *daf-16(mgDf47*) and *age-1(hx546*). All strains were generously shared by the lab of Dr. S.S. Lee (Cornell University, Ithaca, NY). Animals were maintained at 20ºC on petri dishes containing Nematode Growth Medium (NGM) seeded with a live *E. coli* strain OP50 as the food source according to the general procedures outlined by Brenner (137).

2.2.5 Lifespan assay

Several gravid adult nematodes were placed on NGM plates seeded with

E. coli strain OP50 and allowed to lay eggs at 20ºC for approximately 6 hours to obtain a synchronous population. After 6 hours, the nematodes were removed and the plates were placed back at 20ºC until the progeny reached young adulthood (about 72 hours). On day 0 of the experiment, these young adult nematodes were transferred to 35 mm NGM petri dishes containing either no apple extracts or the appropriate doses of dissolved apple extracts and 50 µM of 5-fluoro-2-deoxyuridine (FUDR) to prevent progeny production. Plates were then dried in a sterile hood, seeded with 100 µL of 3-fold concentrated, saturated *E. coli* OP50 culture, and dried again. Animals were transferred every other day to fresh extracts or control plates until day 8 of adulthood. Worms were scored daily or every other day by gentle prodding with a platinum wire. Those animals that failed to move were scored as dead. Animals, which exhibited bagging, exploded or crawled off the plates, were censored. Statistical analyses were performed using SPSS statistical software Kaplan-Meier Survival function; *p*-values were obtained using the log-rank test. The experiment was repeated multiple times and a representative trial is shown. All experiments, except for the *P. aeruginosa* pathogen killing assay and heat shock treatment were performed at 20ºC.

2.2.6 Healthspan assays

2.2.6.1 Lipofuscin

Animals (N=18 per group) were treated as per the lifespan assay described above, picked off the plates on day 8 of adulthood, placed onto 2% agarose pads and immobilized in 20 µM sodium azide (108). Slides were visualized using the Leica DM5000B Microscope (Bannockburn, IL), with the I3 cube filter (excitation 450/490, emission 510), and images were captured

using a Hamamatsu ORCA-ER camera and OpenLab software. Image quantification of fluorescence intensity was done densitometrically by tracing around each animal's intestine and determining average pixel intensity using imageJ freeware (NIH) (135).

2.2.6.2 Motility assay

The effect of apple extracts on motility of the worms was determined by a motility assay adapted from Herndon *et al.* and Golden *et al.* (81, 138). Animals were treated as per the lifespan assay described above. On days 12, 14, 16 and 18 of adulthood, animals were visualized using an Olympus SZ61 stereomicroscope (New York/New Jersey Scientific, Middlebush, NJ). Motility classes were determined using the method reported by Golden *et al*., where 'A' worms move spontaneously and smoothly, leaving sinusoidal and symmetric tracks; 'C' worms only move the nose or tail when prodded with a platinum wire; and 'B' animals represent every behavioral class in-between. N≥47 animals for days 10 and 12, N≥25 for day 16.

2.2.7 Stress resistance assays

For all stress resistance assays, animals were transferred to 35 mm NGM/OP50 plates with apple extracts or control plates at the young adult stage containing 50 µM FUDR, then incubated for two days followed by exposure to the stressor on the third day of adulthood. All trials were repeated at least 2-3 independent times, and a representative trial is shown.

2.2.7.1 Heat shock

Animals were incubated at 35ºC on the third day of adulthood for 8

hours and monitored every 30 minutes thereafter until approximately 50% of the controls had died. Plates were pulled out of the incubator and scored for survival (139). Triplicate plates were used per each time point with N≥61 animals per group.

2.2.7.2 UV irradiation

Animals were transferred to bacteria-free NGM plates on day three of adulthood and UV-irradiated at 1200 J/m^2 with a UV Stratalinker 2400 (Stratagene, La Jolla, CA) equipped with five 254 nm UV light bulbs (model 1800), each generating 15 watts. After UV irradiation, animals were transferred back to the standard NGM/OP50 plates without apple extracts and monitored daily for survival by gentle prodding with a platinum wire (140). N=66, 82, 79, 72 animals for 0, 2.5, 5 and 10 mg/mL groups, respectively.

2.2.7.3 *Pseudomonas aeruginosa* infection

Animals were grown on NGM/OP50 plates at 25ºC and transferred to plates with or without apple extracts for a period of two days. On the third day of adulthood, animals were shifted to modified NGM plates containing *P. aeruginosa* strain PA14 at 25ºC, and scored for survival every 8-13 hours. These modified NGM plates were prepared according to the method of Tan *et al*. (141). Briefly, plates were seeded with 10 µL of *P. aeruginosa*, allowed to dry overnight at 37ºC and then at room temperature for another 24 hours. N=82, 80, 73 and 76 animals for 0, 2.5, 5 and 10 mg/mL groups, respectively.

2.2.7.4 Oxidative stress

The oxidative stress paraquat assay on plates was performed using the

methods described previously (96, 123). On day three of adulthood, animals were transferred to freshly prepared NGM/OP50 plates, containing 10 mM paraquat and scored as above. N≥54 animals per each group.

2.2.8 Brood size

N2 worms were grown on NGM/OP50 plates until the late L4 stage and transferred to the control plate or plates with different concentrations of apple extracts, one worm per plate per concentration, with N=8 animals per group. Animals were then transferred every 24 hours to the fresh control or apple extracts plates until egg production had ceased. Total number of progeny that grew up from each worm was counted, and the number of progeny for each concentration was averaged (142).

2.2.9 Statistical analyses

Survival data were analyzed using SPSS version 16 for Windows (SPSS Inc., Chicago, IL) Kaplan-Meier Survival function and log-rank test. All other analyses were done using Minitab statistical software (State College, PA). Data for heat shock were analyzed using two-sample *t*-test (assuming equal variance); lipofuscin accumulation and brood size were analyzed using one-way ANOVA; motility classes were compared using logistic regression. Graphs were plotted with the SigmaPlot version 10 for Windows software (Systat Software Inc., San Jose, CA). A *p*- value < 0.05 was considered to be statistically significant.

2.3 Results

2.3.1 Effect of apple extracts on wild-type C. elegans lifespan

Wild-type adult *C. elegans* have an average lifespan of 2-3 weeks at 20ºC. Under our standard laboratory conditions, control wild-type animals lived an average of 17.23 **±** .30 days (maximum of 24 days). After administering 2.5, 5 and 10 mg/mL of apple extracts under standard laboratory conditions, starting at the young adult stage, mean lifespan increased to 20.46 ± .17 days (maximum of 24 days), 21.38 ± .35 days (maximum of 30 days) and 24.05 ± . 53 days (maximum of 30 days), respectively, in a significant, dose-dependent manner (Figure 2.1; Table 2.1). These changes represent lifespan increases of 18.7, 24.1, and 39.6 %, respectively, when compared to the control group (Figure 2.2; Table 2.1).

2.3.2 Effect of apple extracts on motility and lipofuscin accumulation

Next, we examined whether the increase in lifespan was accompanied by an overall improvement in health and vitality. To this end, we tested the motility of 12-, 14-, and 16-day-old worms treated with three concentrations of apple extracts. Motility was classified according to movement spontaneity on petri dishes: Class A animals moved spontaneously; class B animals required prodding to stimulate whole-body movement; and class C animals moved only their heads in response to prodding (81, 138). The decline in motility on days 12, 14, and 16 was delayed significantly in a dose-dependent manner in worms treated with low (2.5 mg/mL), moderate (5 mg/mL), and high (10 mg/mL) concentrations of apple extracts (Figures 2.3-2.5). On day 12, most of the animals in all groups continued to move spontaneously, but there was already a small, significant difference in spontaneous (Class A) motility among

the control and treatment groups (Figure 2.3). By day 14, animals treated with low, moderate, and high doses of apple extracts had significantly (p<0.05) more high-motility (class A) individuals (63, 49, and 77%, respectively) than the control group (15%) (Figure 2.4). By day 16, the dose-dependent effects on motility were fully evident (Figure 2.5), such that 92% of the control group would barely move their heads with prodding (class C), while 49% of the high-dose treatment group still moved spontaneously (class A). We continued to follow the animals to day 18, when most of the control animals had died, treatment groups still displayed highly significant dose-dependent differences in motility (data not shown).

Lipofuscin is a byproduct of lysosomal degradation that accumulates with age in many organisms, including *C. elegans* (143). Wild-type N2 worms treated with 2.5 and 5 mg/mL apple extracts accumulated only about half as much lipofuscin as the N2 control worms (Figure 2.6). *daf-16* nematodes, which age and accumulate lipofuscin at a faster rate than wild-type N2 worms, were used as a positive control. As expected, they accumulated more lipofuscin after 8 days than did control animals of N2.

2.3.3 Effects of apple extracts on resistance to heat stress, UV irradiation and pathogenic infection

Increased lifespan often correlates with increased stress resistance (120, 144). We treated young adult *C. elegans* with doses of 0, 2.5, 5 and 10 mg/mL apple extracts for two days, and examined their response to a variety of chemical and environmental stressors, including heat shock, UV radiation, and the pathogen *Pseudomonas aeruginosa*.

Animals were placed in a 35ºC heat shock chamber and monitored until

approximately half (55%) of the wild-type N2 controls had died (10.5 hours). At that time, 71.4, 87.1 and 89.73% of 2.5, 5 and 10 mg/mL apple-treated animals were still alive, respectively. *age-1* worms, which are more resistant to heat stress, were used as a negative control and survived at a 96.13% rate; *daf-16* worms, which are more sensitive to heat stress, were used as a positive control and had a 28.96% survival rate (Figure 2.7)

In an analogous experiment, N2 worms pretreated with apple extracts survived significantly longer after UV irradiation than N2 control worms (Figure 2.8; Table 2.2). Post-irradiation survival time for N2 worms pretreated with 2.5, 5 and 10 mg/mL apple extracts was increased by 22.6, 46.2, 61.5%, respectively. *age-1* worms, which are more resistant to UV stress than N2, were used as a negative control and survived 74.4% longer than the N2 controls. *daf-16* worms, which are more sensitive to UV stress than N2, were used as a positive control and survived 12.8% less than the N2 controls.

N2 worms treated with apple extracts showed increased resistance to the pathogen *Pseudomonas aeruginosa*. After being transferred to pathogen-seeded plates on the third day of adulthood and monitored every 8-13 hours, control worms survived an average of 82 ± 2.6 hours, while worms treated with 2.5, 5 and 10 mg/mL of apple extracts had significantly increased mean lifespans of 100 ± 3.8, 111 ± 4.4 and 102 ± 4.4 hours, respectively (Figure 2.9; Table 2.3).

2.3.4. Effects of apple extracts on resistance to oxidative stress induced by paraquat

To test the antioxidant effects of apple extracts, we placed worms on NGM plates containing 10 mM of the superoxide-generating chemical

paraquat. While control worms survived 4.97 ± 0.19 days (maximum of 7 days), worms treated with 2.5, 5 and 10 mg/mL of apple extracts survived for 7.71 ± 0.32 (maximum of 10 days), 9.41 ± 0.43 (maximum of 14 days) and 8.54 ± 0.39 days (maximum of 15 days), respectively. These represented statistically significant increases in mean life span of 55.1, 89.3 and 71.8% over the control (Figure 2.10; Table 2.4).

2.3.5 Effect of apple extracts on brood size

To see if apple extracts adversely affected fecundity, brood sizes were measured for 8 N2 worms per each group: 0 mg/mL (control), 2.5, 5 and 10 mg/mL. There were no significant differences between treatment groups and the control (Figure 2.11).

Table 2.1 Effects of apple extracts on mean lifespan of *C. elegans.*

Treatment	N	Mean Lifespan* (days)	Δ**	*p*-value vs. control	% of Control
0 mg/mL (control)	120	17.23±.30	a	N.A.	100.0
2.5 mg/mL	132	20.46±.17	b	<0.001	118.7
5 mg/mL	158	21.38±.35	c	<0.001	124.1
10 mg/mL	120	24.05±.53	d	<0.001	139.6

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

Table 2.2 Effect of pretreatment with apple extracts on resistance to UV irradiation in *C. elegans*.

Treatment	N	Mean Lifespan* (days)	Δ**	*p*-value vs. control	% of Control
0 mg/mL (control)	66	3.92±.15	a	N.A.	100.0
2.5 mg/mL	82	4.88±.18	b	<0.001	122.6
5 mg/mL	79	5.70±.18	c	<0.001	146.2
10 mg/mL	72	6.37±.18	c,d	<0.001	161.5
age-1 (neg control)	36	6.89±.32	d	<0.001	174.4
daf-16 (pos cont)	38	3.49±.19	e	<0.001	87.2

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

Table 2.3 Effect of pretreatment with apple extracts on resistance to infection by *Pseudomonas aeruginosa* in *C. elegans.*

Treatment	N	Mean Lifespan* (hours)	Δ**	*p*-value vs. control	% of Control
0 mg/mL (control)	82	82.0±2.62	a	N.A.	100.0
2.5 mg/mL	80	100.0±3.75	b	<0.001	122.0
5 mg/mL	73	110.9±4.41	c	<0.001	135.2
10 mg/mL	76	102.1±4.4	b,c	<0.001	124.5

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

Table 2.4 Effect of pretreatment with apple extracts on resistance to chronic challenge with paraquat in *C. elegans.*

Treatment	N	Mean Lifespan* (days)	Δ**	*p*-value vs. control	% of Control
0 mg/mL (control)	67	4.97±.19	a	N.A.	100.0
2.5 mg/mL	58	7.71±.32	b	<0.001	155.13
5 mg/mL	54	9.41±.43	c	<0.001	189.3
10 mg/mL	61	8.54±.39	c	<0.001	171.83

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

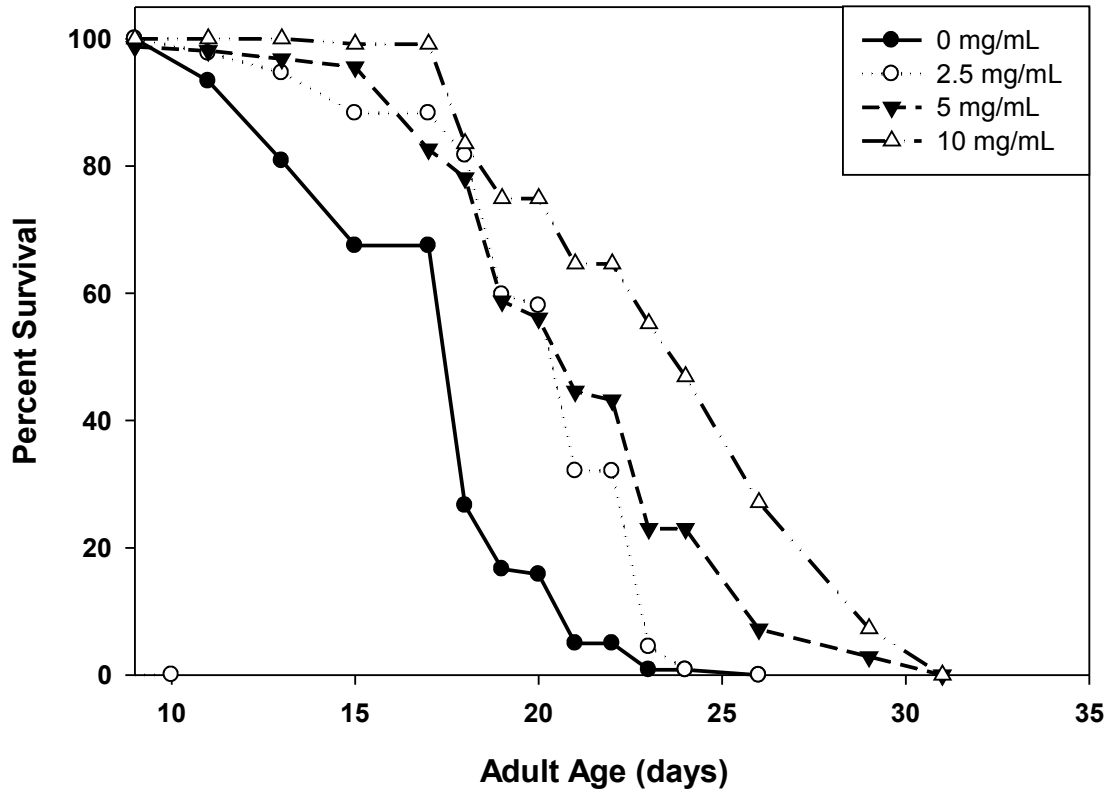


Figure 2.1 Effect of apple extracts on the lifespan of *C. elegans.* Day 1 young adult wild-type animals (N ≥120 in each group) were treated without (0 mg/mL) or with a low (2.5 mg/mL), moderate (5 mg/mL) and high (10 mg/mL) dose of standardized apple extracts, which contained 170 ± 4.6 mg of phenolics per 100 g of apples. Survival was monitored starting on day 1 of adulthood. Nematodes, that were exposed to three levels of apple extracts survived significantly longer than those that did not (p<0.001 for 0 mg/mL compared to each treatment group; log-rank test; see Table 2.1) The experiment was repeated multiple times and a representative trial is shown.

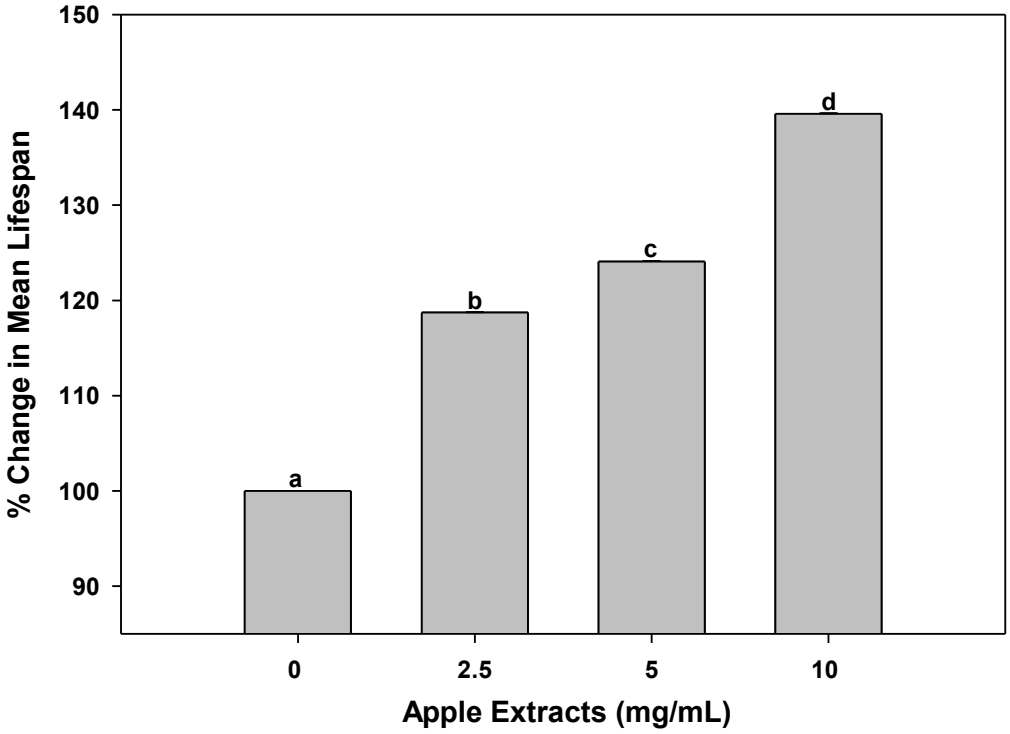


Figure 2.2 Effect of apple extracts on the mean lifespan of *C. elegans.* Bars with no letters in common represent groups that are significantly different (see Table 2.1).

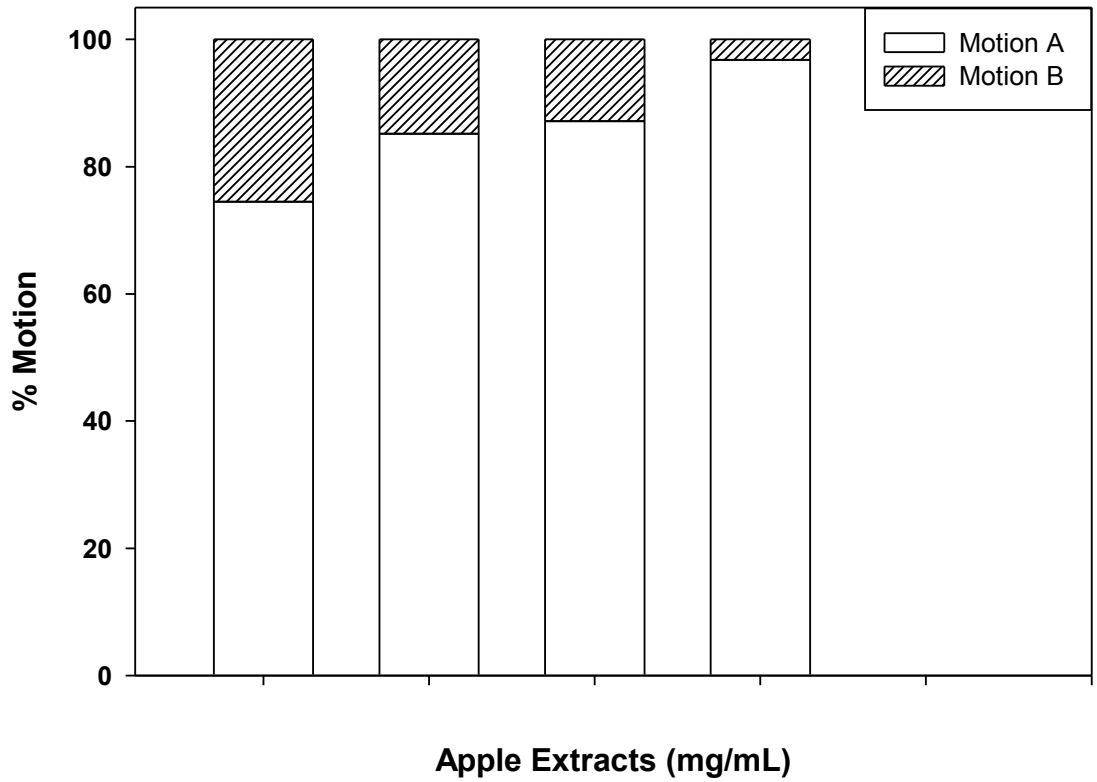


Figure 2.3 Effect of apple extracts on motility of *C. elegans* treated on day 12. Motility was classified into two groups: Motion A animals moved spontaneously; Motion B animals required prodding to stimulate movement. The decline in motility on day 12 was significantly delayed in a dose-dependent manner in worms treated with 2.5, 5, and 10 mg/mL of apple extract (N ≥ 47 animals per group; binary logistic regression; p=0.001; likelihood ratio test statistic G=12.051 with df1). A p-value of < 0.05 was considered to be statistically significant.

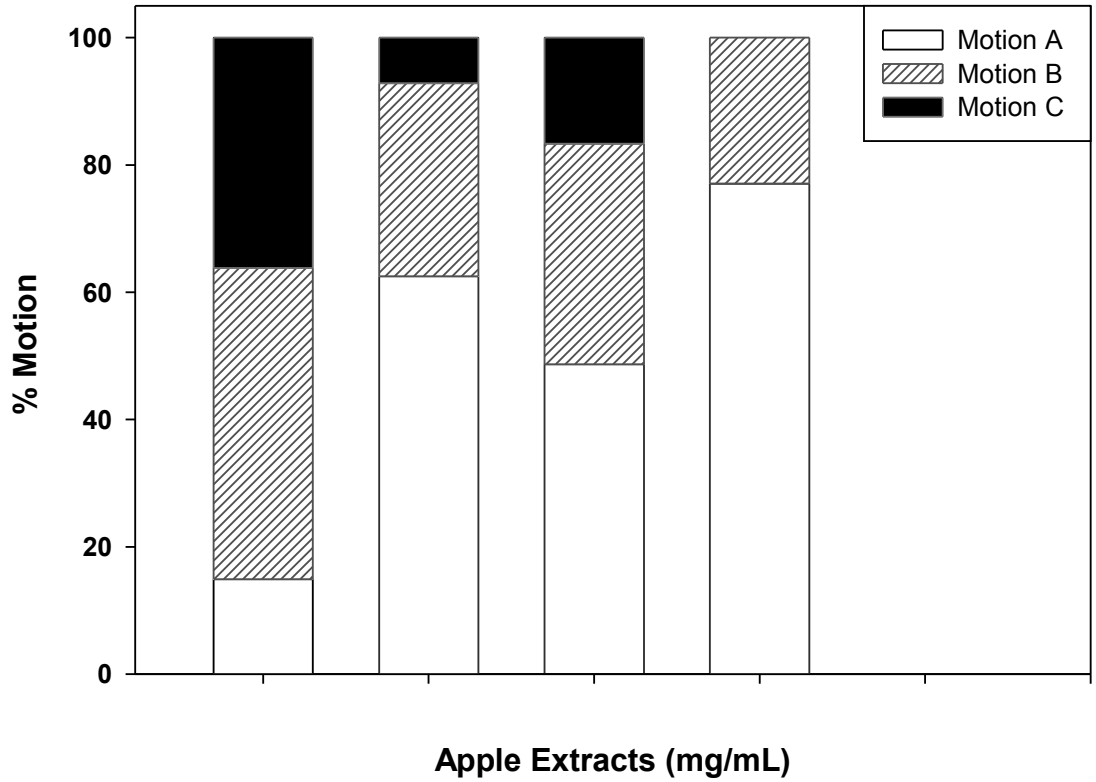


Figure 2.4 Effect of apple extracts on motility of *C. elegans* treated on day 14. Motility was classified into three groups: Motion A animals moved spontaneously; Motion B animals required prodding to stimulate movement; and Motion C animals only moved their heads in response to prodding. The decline in motility on day 14 was significantly delayed in a dose-dependent manner in worms treated with 2.5, 5, and 10 mg/mL of apple extract (N ≥ 47 animals per group; nominal logistic regression, with two slope parameters; p=0.001; likelihood ratio test statistic G=39.1 with df2). A p-value of < 0.05 was considered to be statistically significant.

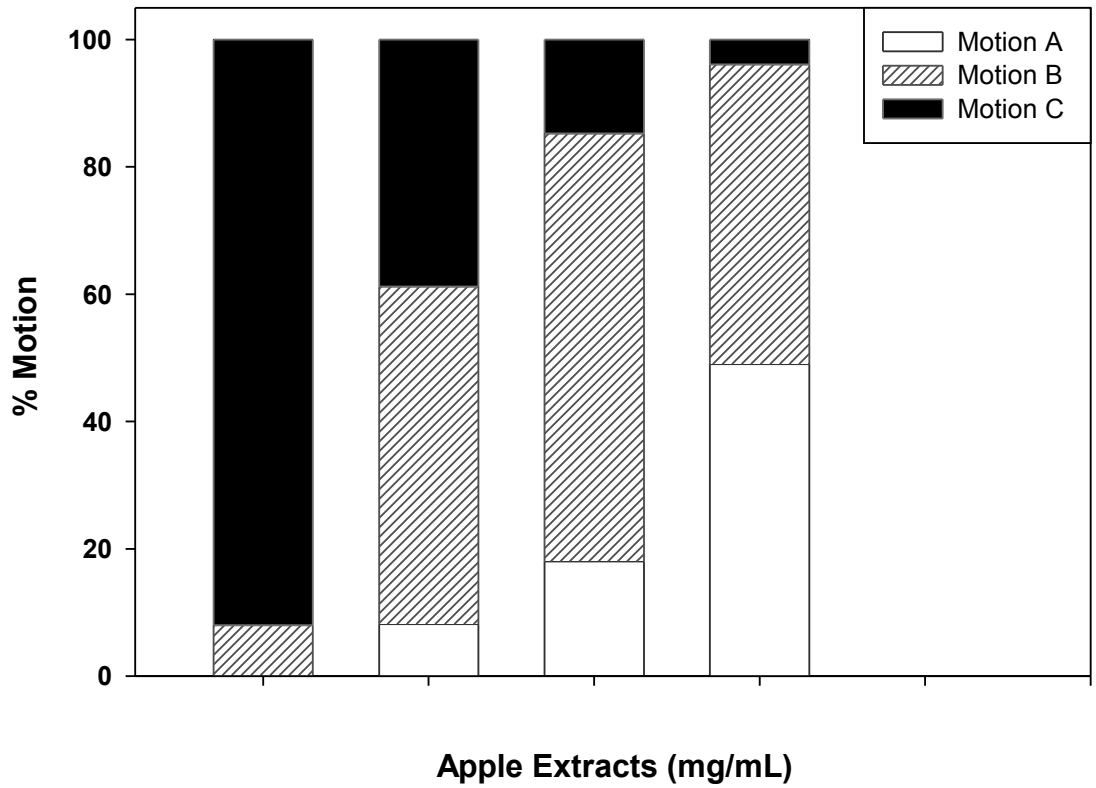


Figure 2.5 Effect of apple extracts on motility of *C. elegans* treated on day 16. Motility was classified into three groups: Motion A animals moved spontaneously; Motion B animals required prodding to stimulate movement; and Motion C animals only moved their heads in response to prodding. The decline in motility on day 16 was significantly delayed in a dose-dependent manner in worms treated with 2.5, 5, and 10 mg/mL of apple extract (N ≥ 25 animals per group; nominal logistic regression, with two slope parameters; p=0.001; likelihood ratio test statistic G=84.3 with df2). A p-value of < 0.05 was considered to be statistically significant.

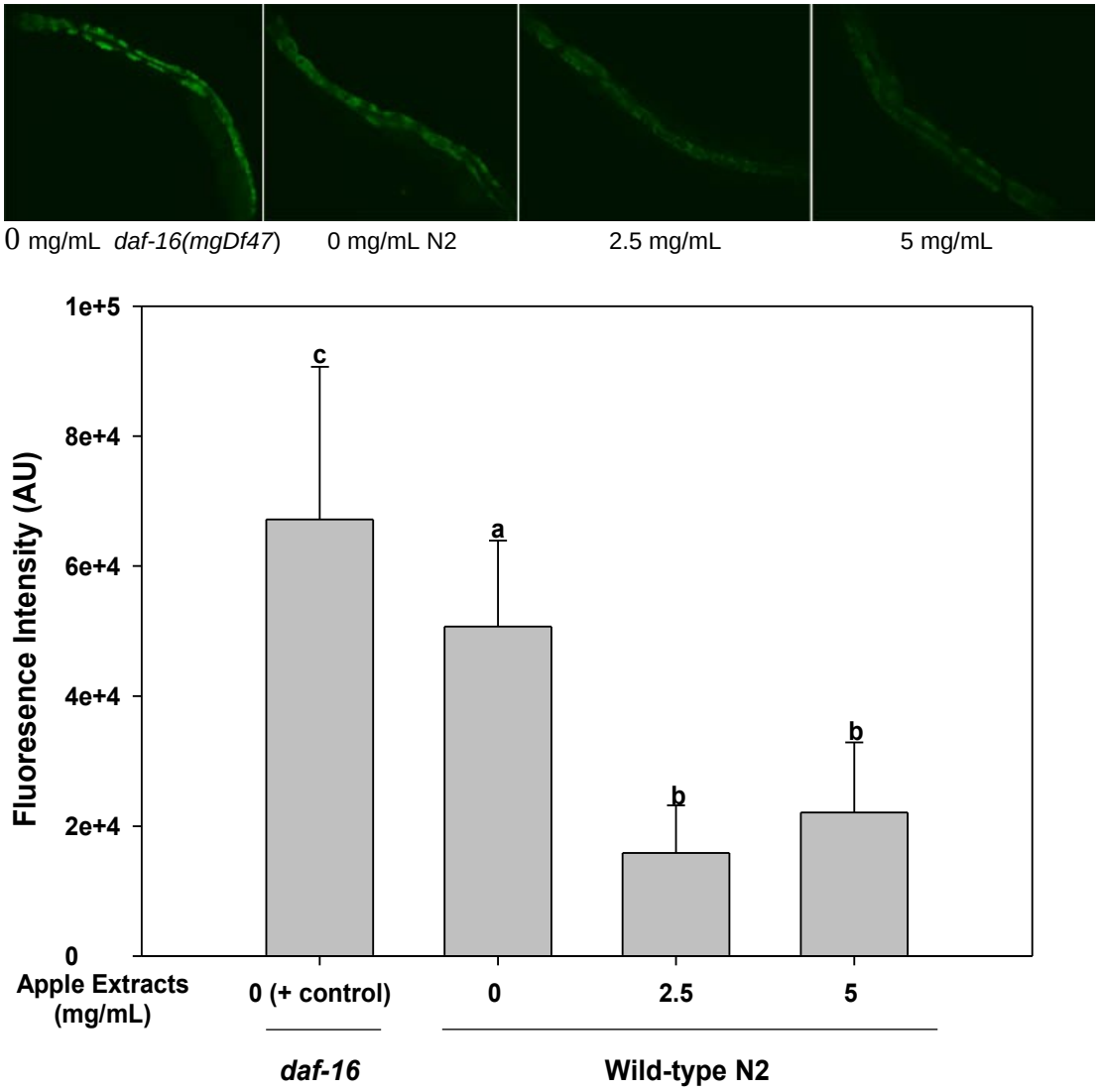


Figure 2.6 Effect of apple extracts on lipofuscin accumulation in *C. elegans.* Wild-type N2 worms treated with 2.5 and 5 mg/mL apple extracts accumulated only about half as much lipofuscin as the N2 control worms (p<0.001 for 0 vs. all other groups; One-way ANOVA, Tukey's multiple comparison test for differences among groups; see Appendix II). A p-value of < 0.05 was considered to be statistically significant. *daf-16* nematodes without extract treatment, which age at a faster rate, were used as a positive control. Bars with no letters in common are significantly different. N=18 animals per group.

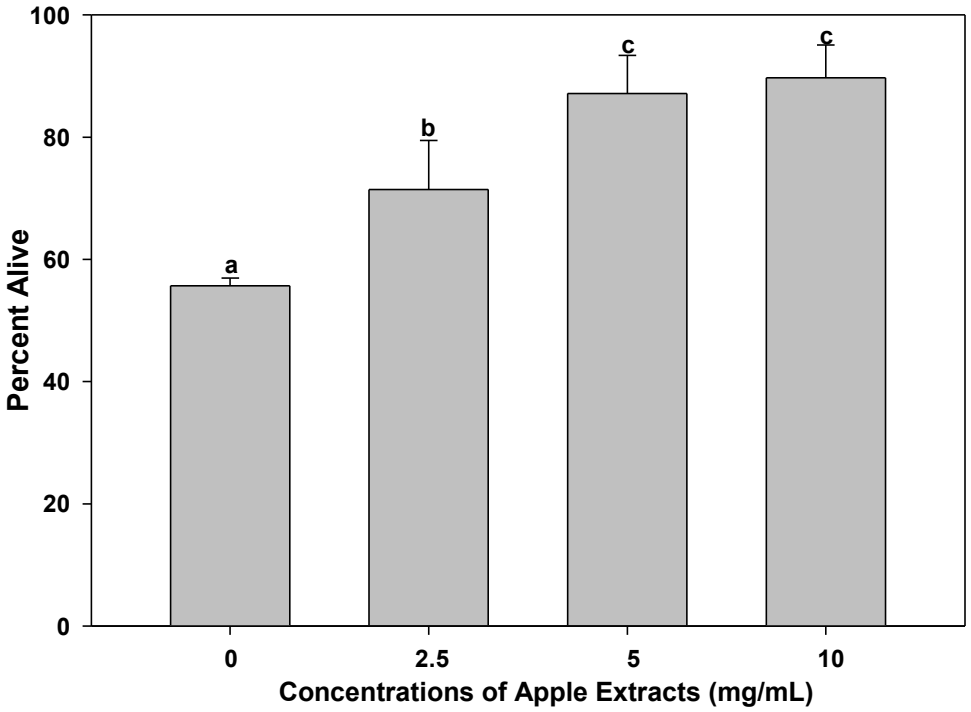


Figure 2.7 Effect of pretreatment with apple extracts on resistance to heat shock in *C. elegans*. Animals were treated with various concentrations of apple extracts (0, 2.5, 5, or 10 mg/mL, in each group) as young adults for 2 days at 20°C and exposed to a variety of stressors on the third day of adulthood. Nematodes, that were pretreated with apple extracts, survived significantly longer after 35°C heat shock. Bars with no letters in common are significantly different. N ≥ 61 animals per group. Significance was determined using One-way ANOVA, Tukey's multiple comparison test for differences among groups; see Appendix II. A *p*-value of < 0.05 was considered to be statistically significant).

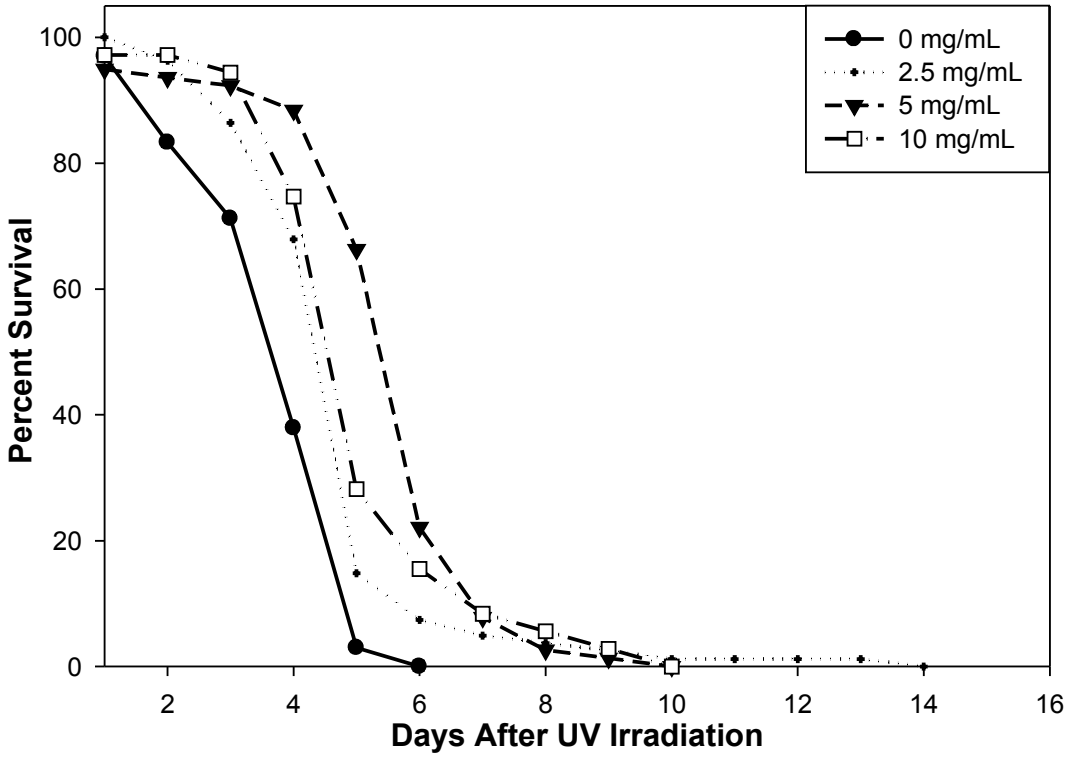


Figure 2.8 Effect of pretreatment with apple extracts on resistance to UV-irradiation in *C. elegans*. Animals were treated with various concentrations of apple extracts (0, 2.5, 5, or 10 mg/mL, in each group) as young adults for 2 days at 20°C and exposed to a variety of stressors on the third day of adulthood. Nematodes, that were pretreated with apple extracts, survived significantly longer after UV irradiation at 1200 J/m^2. N ≥ 66 animals per group. Animals that were exposed to three levels of apple extracts survived significantly longer than those that did not (p<0.001 for 0 mg/mL control versus each of the treatment groups; log-rank test; see Table 2.2). A p-value < 0.05 was considered to be significantly different. The experiment was repeated multiple times and a representative trial is shown.

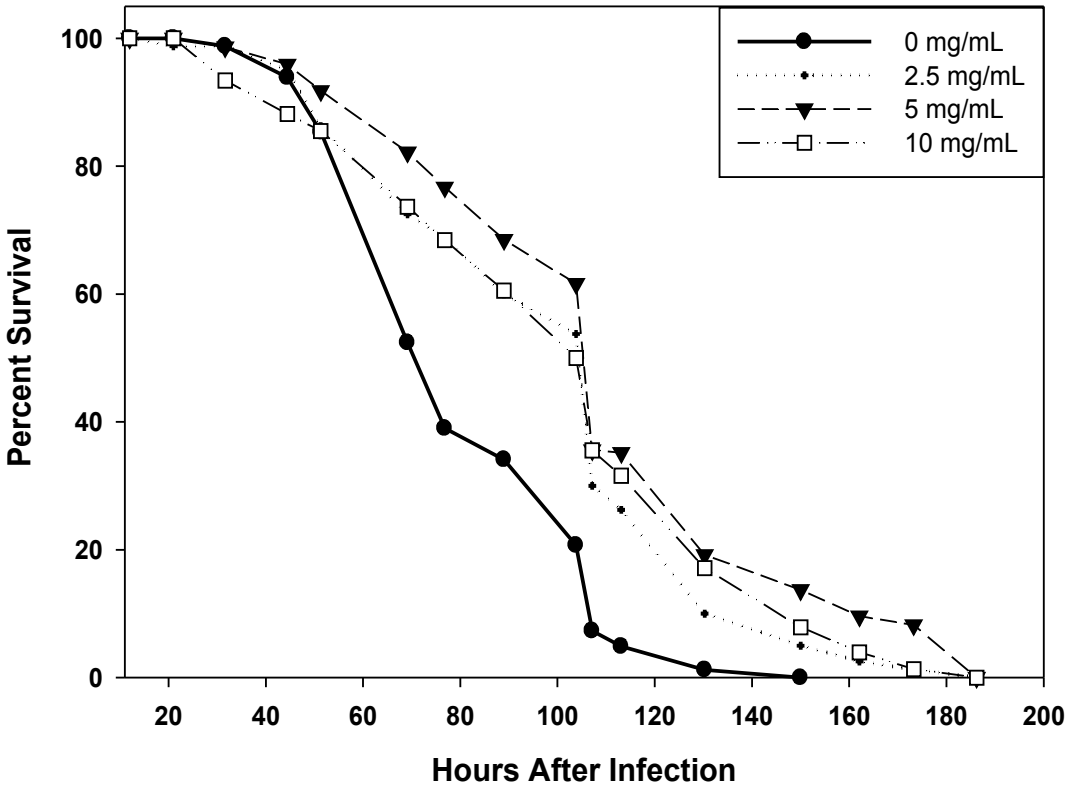


Figure 2.9 Effect of pretreatment with apple extracts on resistance to pathogenic infection in *C. elegans*. Animals were treated with various concentrations of apple extracts (0, 2.5, 5, or 10 mg/mL, in each group) as young adults for 2 days at 20°C and infected with *P. aeruginosa* on the third day of adulthood. Animals, that were pretreated with three levels of apple extracts survived significantly longer after infection (N ≥ 73 animals per group, $p<0.001$ control versus each treatment group, log-rank test; see Table 2.3). A p-value < 0.05 was considered to be significantly different. The experiment was repeated multiple times and a representative trial is shown.

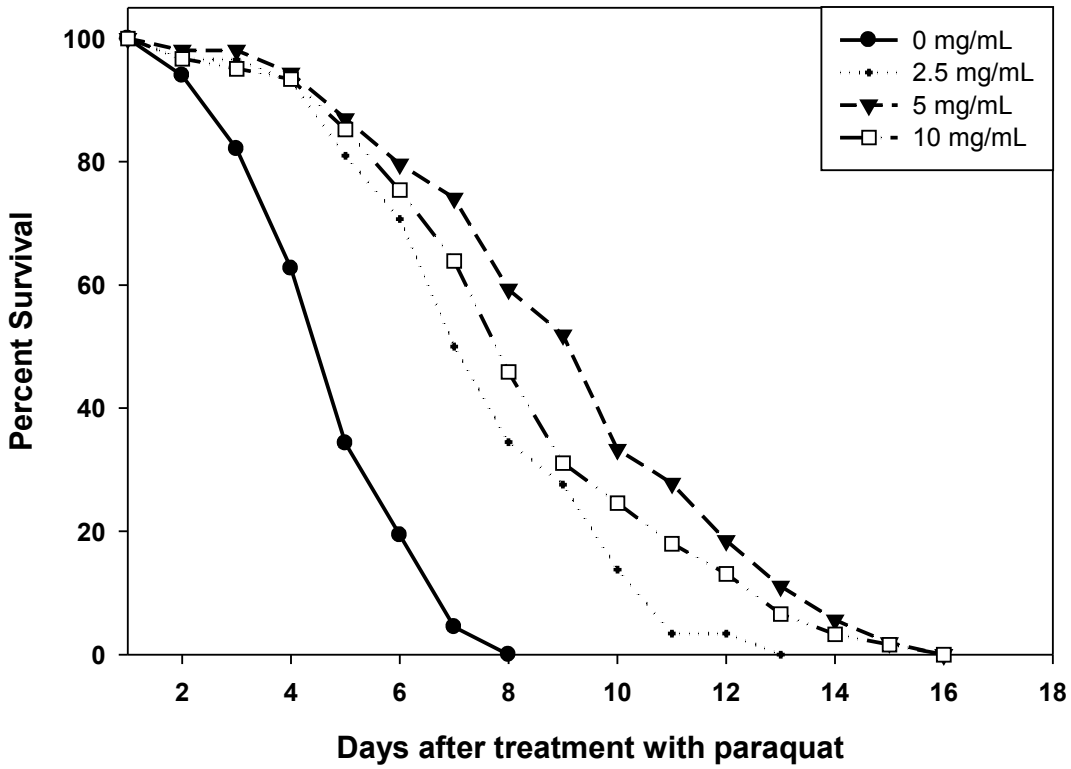


Figure 2.10 Effect of pretreatment with apple extracts on resistance to paraquat-induced oxidative stress in *C. elegans*. Animals were treated with various concentrations of apple extracts (0, 2.5, 5, or 10 mg/mL, in each group) as young adults for 2 days and transferred to NGM plates containing 10mM paraquat on the third day of adulthood. Animals, that were pretreated with three levels of apple extracts survived significantly longer (N ≥ 73 animals per group, $p<0.001$ control versus each treatment group, log-rank test; see Table 2.4). A p-value < 0.05 was considered to be significantly different. The experiment was repeated multiple times and a representative trial is shown.

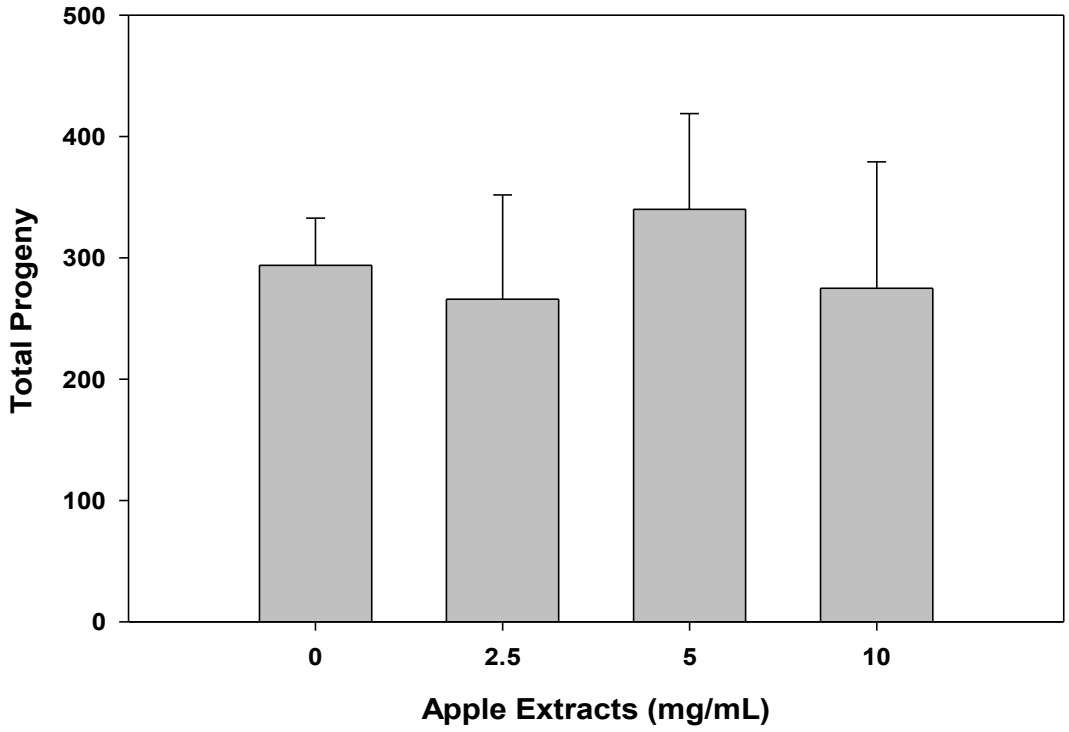


Figure 2.11 Effect of apple extracts on brood size of *C. elegans.* N=8 animals per group. There were no significant differences in total progeny output between animals that were treated with or without apple extracts (p=.321, One-way ANOVA). A p-value < 0.05 was considered to be significantly different.

2.4 Discussion

Multiple studies have shown that compounds and extracts derived from plants, and in particular plant foods, possess a wide range of health benefits including chemoprevention, anti-oxidation, anti-proliferation and modulation of a variety of enzymes and transcription factors (4). However, until fairly recently direct anti-aging effects of these compounds and extracts had not been observed. Kitani *et al.* showed that green tea polyphenols can increase the lifespan of mice by up to 72.6%, and suggested that dietary approaches to increasing lifespan, especially those that possess antioxidant effects, warrant further investigation (145). Casadesus and colleagues found that a diet supplemented with blueberries improved spatial memory and increased parameters associated with hippocampal neuronal plasticity (34). Similarly, Joseph *et al.* proposed that berry fruits could slow brain aging by lowering oxidative stress and inflammation, modulating cell signaling involved in neuronal communication and neuroprotective stress shock proteins, and buffering calcium levels (33). In *C. elegans*, Wilson *et al.* showed that supplementation of the nematode diet with blueberry extracts extends lifespan via the CaMKII pathway and improves thermotolerance (108). Perhaps the most familiar example of a plant-derived compound with anti-aging properties from recent history is resveratrol, a phytochemical found in the skins of grapes, red wine, peanuts and other plant foods. Resveratrol has been shown to increase lifespan under certain conditions in multiple species via mechanisms that may involve a wide spectrum of targets (89, 125, 128, 146).

In the current study, we show for the first time that phytochemicals from apples, one of the most commonly consumed fruits, can extend lifespan in a dose-dependent manner in *C. elegans*. Our findings indicate that *C. elegans*

can live up to 39% longer when fed a diet modified with apple extracts. We suggest that the observed anti-aging effects are due to additive and synergistic effects of many bioactive compounds found in apples, as we proposed earlier (10). Apple phytochemicals possess a variety of bioactivities, including antioxidant, anti-proliferative and anti-inflammatory activity, inhibition of NF-kB, modulation of cell signaling via MAP kinases and PI3K pathways, induction of apoptosis, and regulation of the cell cycle via G1 arrest and checkpoint proteins such as Cyclin D1 and Cdk4 (11, 39, 40, 49, 50, 52, 55, 147). Given this plethora of effects, it is likely that apple phytochemicals act on several distinct pathways to extend lifespan. Additionally, quercetin and EGCG, two phytochemicals that are found in apples, have been shown to extend lifespan in *C. elegans* (130, 148-150). However, the magnitude of lifespan extension for these single phytochemicals (up to 15% for quercetin and either 0, 5 or 10% for EGCG depending on the dose and culturing conditions) was not as great as that which we observed in animals treated with whole apple extracts, suggesting that interaction among various compounds is needed to elicit the maximum benefits (10). These results are also in agreement with our own preliminary data, which show that quercetin-3-β-D-glucoside and 2-α-hydroxy-ursolic acid, two of the major phytochemicals contained in apples, were not as potent in increasing *C. elegans* lifespan as were whole apple extracts (data not shown).

In a recent study using blueberry extracts, Wilson *et al.* reported that lifespan and thermotolerance of *C. elegans* were significantly increased by up to 28% and 2.5-fold, respectively (108). Interestingly, the authors did not detect a dose-dependent response, nor did they observe any antioxidant effects upon induction of mild to severe oxidative stresses, as was previously

reported in other *in vitro* and *in vivo* models (39, 151, 152). Instead, the effects appeared to be mediated by the CaMKII pathway, which modulates osmotic stress resistance. Separation of the blueberry extracts revealed that of the three major fractions only the proanthocyanidin fraction, and not the anthocyanin or chlorigenic acid fractions, contributed to an increase in lifespan. This is to some extent surprising because a recent meta-analysis of 97 studies on bioavailability revealed that, at least in humans, proanthocyanidins are the least well-absorbed polyphenols (153). Perhaps in *C. elegans* these compounds have greater bioavailability or are necessary in much smaller amounts to elicit their beneficial effects. In contrast, the same meta-analysis revealed that isoflavones, catechins and derivates of quercetin, which are among the most abundant polyphenols found in apples, are highly absorbed. An increase in lifespan often comes at the expense of reduced fertility. In our study, we examined the fecundity of the animals and found that the total amount of eggs laid did not differ among the treatment and control groups. In the blueberry study, because the researchers used a sterile strain as a control, such an assessment was not possible. However, pump rate was not adversely affected and actually improved, suggesting that the doses tested were not toxic and the effects were not due to calorie restriction.

Another study, by Weigant *et al.* reported that plant adaptogens of the extracts of *Eleutherococcus senticosus* and *Rhodiola rosea* increased mean lifespan in a dose-dependent manner by over 15%. The researchers determined that *daf-16*, a FOXO-like transcription factor responsible for activating many genes involved in cellular stress response, metabolism and longevity, translocated to the nucleus upon treatment. In addition, they observed the induction of GFP fused to the heat shock promoter. The authors

concluded that plant adaptogens may be acting as hormetic agents that exert their benefits by inducing mild stress (109).

In addition to living longer, the animals in our study showed an improved quality of life. Healthspan was measured by two biomarkers, lipofuscin and motility. Lipofuscin, a fluorescent pigment that accumulates with age in many species including humans, was significantly attenuated in animals that were pretreated with apple extracts. Apple extracts-treated groups showed a two-fold decrease in the accumulation of lipofuscin (Figure 2). Motility was also improved following treatment with apple extracts. After dividing animals into three groups, 'A' – spontaneous movement, 'C' –no movement but still alive, and 'B' – every state of movement in between 'A' and 'C,' we found that nematodes treated with apple extracts consistently showed more youthful and vigorous movement than control animals. The biggest change was observed in animals on days 14 and 16 (roughly equivalent to 45-55 and 55-65 human years of age). We continued to follow apple extracts-treated animals through day 18 (roughly humans aged at 75-85 years) even though more than half of the control animals had already died. Interestingly, we observed that the motility of many animals in apple-treated groups on day 18 were still in classes 'B' and 'A' (data not shown).

In addition to examining a physical behavior, motility, and a physiological characteristic, lipofuscin accumulation, we tested the animals' fitness by examining responses to various stressors. Previous studies have established a correlation between longer lifespan and resistance to stresses in *C. elegans* and other animals, including mammals (120, 144, 154). After pretreatment with low, moderate and high doses of apple extracts for the first two days of adulthood, the animals were challenged with a battery of stressors

including heat shock, UV radiation, paraquat-induced oxidative stress, and the pathogen *P. aeruginosa.* We removed the animals from the apple extracts plates following the second day to ensure that the effects we observed were preventative, as well as to eliminate the possibility of a confounding effect between the stressors and treatments.

Pretreatment with apple extracts greatly improved survival following heat shock. This result is consistent with other studies in *C. elegans* that observed enhancement of thermotolerance by plant-derived extracts (108, 121). Apple extracts may have affected stress signaling pathways via modulation of heat shock proteins (*hsps*) (121). The extracts may have acted as mild stressors and affected the activity of these or other stress proteins, which promote a generalized, protective stress response. This might explain why animals that were pre-exposed to apple extracts had a significantly improved survival. Consistent with this hypothesis, data from other experiments, which examined *C. elegans* survival and stress response, indicates that beyond their antioxidant properties phytochemicals may possess hormetic effects that confer a generalized cellular protective response when administered at low doses (109).

Survival following UV irradiation, which may mimic damage by the sun, was also significantly improved in our study after pretreatment with apple extracts. UV damage is known to cause skin cancer, accelerate aging, and damage DNA. For example, cyclobutane pyrimidine dimers (CPDs) and pyrimidine pyrimidone photoproducts are produced after irradiation with UVC (200-280 nm) light. DNA repair mechanisms are critical for repairing this damage. Natural products that may stimulate these mechanisms include resveratrol and curcumin, which were shown to accelerate DNA repair

efficiency and unscheduled DNA synthesis in cultured Chinese hamster lung fibroblast cells (155). Another possible mechanism by which apple extracts may be preventing or repairing UV damage, is regulation at the transcription factor level. For example, UVC damage is known to activate transcription factor NF-kB (156), which mediates the transcription of many genes including those that are involved in cell proliferation, cell survival, and inhibition of apoptosis. Expression of these genes may lead to tumorigensis. Previous results from our lab have shown that NF-κB was inhibited by 2-α-hydroxy-ursolic acid, one of the major phytochemical components found in apple extracts (52).

Aging is associated with an increase in oxidative damage to DNA, proteins, and lipids (65). We examined the effects of apple extracts on paraquat-induced oxidative stress in *C. elegans*. After exposing the animals to paraquat (10 mM), a superoxide-generating chemical, we found that their survival was significantly improved by pretreatment with apple extracts in a dose-dependent manner. A superoxide radical is highly reactive and can cause damage to lipid membranes, proteins and DNA. Because paraquat is regenerated via redox cycling, its *in vivo* effects can persist as it continuously gets reduced and oxidized via electron donors and acceptors to produce superoxide. Apple extracts, which as we previously showed possess antioxidant activity, may quench the radicals to prevent damage and thereby improve survival. This finding may hint at a possible antioxidant mechanism of action behind the apple extracts' life extending properties. Further studies are needed to clarify the role of this antioxidative effect.

Animals were also infected with the pathogen *P. aeruginosa* to test their response to pathogenic attack. Infection occurs by ingestion and, under

normal conditions, *P. aeruginosa* is lethal within 1-3 days. Our results indicate that animals pretreated with apple extracts survived up to 35.2% longer, hinting at another possible mechanism of lifespan extension – modulation of the immune response. In *C. elegans*, immune response to this Gram-negative bacterium is mediated by three major pathways: MAP kinase, *dbl-1,* and *daf-2/ daf16* (157). Inactivation of *pmk-1,* one of three kinases of the MAP kinase pathway, is known to increase susceptibility to infection via upstream signaling by the other two MAP kinases, *nsy-1* and *sek-1* (158). In RAW 264.7 macrophages, the apple phytochemical quercetin inhibited MAP kinase activity[81]. Specifically, ERK1/2 phosphorylation and p38 MAPK activity were blocked upon pretreatment with quercetin (159). One way to test if apple extracts act by the same mechanism in *C. elegans* would be to pretreat the *pmk-1* mutants with apple extracts and then expose them to *P. aeruginosa.* If the apple extracts are acting via the MAP kinase pathway, this mutant would be expected to live longer than the untreated *pmk-1* control. Alternatively, the apple extracts may be affecting other pathways that modulate innate immunity such as db*l-1*, which encodes one of four TGF-β-like ligands, or *daf-2/daf16*, the insulin signaling pathway, which also regulates longevity.

We have shown that *C. elegans* pretreated with apple extracts had increased resistance to heat, UV irradiation, oxidative stress and pathogenic attack, suggesting that in this organism, in addition to lifespan and health, cellular defense and immune system functions are also improved.

2.5 Conclusion

We have shown for the first time that apple phytochemical extracts increase lifespan *in vivo* using the *C. elegans* aging model. Our results

indicate that treatment with apple extracts also improves healthspan, as shown by a reduction in lipofuscin accumulation and improved motility. This indicates that apple extracts not only extend the lifespan, but also improve the quality of life in this model. In addition, animals were more resistant to heat, UV radiation, paraquat-induced oxidative stress, and pathogenic infection with the pretreatment of apple extracts, suggesting that cellular defense and immune system functions are also improved. Our findings underscore the importance of consuming a diet that is rich in fruits and vegetables, including apples, rather than single supplements. Future studies are needed to elucidate the mechanisms of action, as well as to determine the effects of the individual phytochemicals that may be responsible for the health benefits of apple extracts.

2.6 Acknowledgements

We thank Carolle Mok for technical assistance, Ben Hamilton, Atsushi Ebata, Nicole Liachko and Gizem Rizki for training and experimental troubleshooting, as well as Jason and David Neuswanger for critical editing of the manuscript.

CHAPTER 3

Apple phytochemicals reduce oxidative stress and improve cellular stress response in *C. elegans*

3.1 Introduction

Epidemiological studies have consistently shown that diets rich in fruits and vegetables are associated with the prevention of age-associated chronic diseases such as cancer, cardiovascular disease and type 2 diabetes (1-3). Apples are among the most highly consumed fruits in the U.S. and are the number one source of fruit phenolics (39). This diverse group of phytochemicals found in a variety of plant foods has been shown to possess significant health benefits (10). Previous work from our laboratory has shown that apple phytochemical extracts have high antioxidant and anti-proliferative activity against colon, liver and breast cancer cells (49, 50). In addition, these extracts prevented DMBA-induced mammary tumors in rats, induced G1 arrest and decreased the expression of Cyclin D1 and Cdk4 in MCF-7 breast cancer cells (11, 55)

Recently, we reported that apple phytochemical extracts extend lifespan in the nematode *C. elegans* by up to 39% in a dose-dependent manner. Moreover, we found that two aging markers, lipofuscin and motility were improved upon treatment, and that apple phytochemical extracts-treated animals showed enhanced resistance to stresses such as heat shock, UV irradiation, pathogenic infection and oxidation.

C. elegans is a popular model in aging research because worms experience many behavioral and physiological declines similar to higher mammals, including humans (77). As they grow older, worms move more

slowly and exhibit sarcopenia - the progressive deterioration of muscle tissue. They also become infertile, and accumulate oxidized proteins and lipofuscin, both of which are hallmarks of aging in most species. In *C. elegans*, standard assay conditions to study the pharmacology of drugs and interactions with genes have been described (105). To date, a variety of human pharmacological interventions ranging from vitamins and clinical drugs to dietary supplements and phytochemicals have been shown to extend lifespan or delay physiological aging and age-related degeneration in *C. elegans* (61, 106). Whole-plant phytochemical extracts have also been tested and found to have a significant life-extending effect (107-109). Wilson *et al.* showed that in *C. elegans*, treatment with blueberry extracts increases thermotolerance and extends lifespan by up to 28%. Several other studies looked at single phytochemicals found in plant foods, including apples. For example, quercetin, EGCG, and resveratrol have been shown to extend lifespan in *C. elegans* under some conditions by a variety of molecular mechanisms (89, 130, 148-150, 160). Previously, we reported that apple phytochemical extracts extended lifespan in *C. elegans* in a dose-dependent manner without toxicity or negatively affecting fecundity at the doses tested. The objective of the present study was to determine the mechanisms of action by which apple phytochemical extracts increase lifespan and improve stress resistance using the *C. elegans* model.

3.2 Materials and methods

3.2.1 Chemicals, reagents and antibodies

Acetone and paraformaldehyde (36%, histology grade) were purchased from Fischer Scientific (Pittsburgh, PA). 5-Fluoro-2-deoxyuridine (FUDR) and

methyl viologen dichloride hydrate (paraquat) were purchased from Sigma-Aldrich (St. Louis, MO). Collagenase type IV was purchased from Sigma-Aldrich (St. Louis, MO). Anti-4-hydroxy-2-nonenal monoclonal antibody was purchased from Genox Corporation (Baltimore, MD). Alexaflour 546 goat-anti-mouse antibody was purchased from Invitrogen Corporation (Carlsbad, CA).

3.2.2 Extraction of apple phytochemicals

Fresh apples of the Red Delicious variety were purchased from Cornell Orchards (Cornell University, Ithaca, NY) and extracted using the method previously reported by our laboratory (50). Briefly, whole apples were sliced, blended in ice-chilled 80% acetone, homogenized and rotary evaporated under vacuum at 45°C until approximately 90% of the filtrate had been evaporated. The concentrate was re-suspended in water, and no other solvent remained in the extract. The extracts have been characterized based on bioactivity-guided fractionation and structure identification using HR-MS, 1D and 2D NMR, and X-ray diffraction analysis using the method we reported previously (40). Apple phytochemical extracts were frozen at –80^{o}C until use.

3.2.3 Strains, maintenance and culturing of nematodes

Strains of *C. elegans* used were: Bristol N2 (wild-type), *daf-16(mgDf47*), *sir2.1(ok434), eat-2 (ad465), mev-1 (kn1), glp-1 (e2141),* CL2070 [dvIs70(*hsp-16.2*::gfp; rol-6(su1006))], CL2166 [dvIs19(pAF15(*gst-4*::GFP::NLS))], and CF1553 [muIs84[pAD76(*sod-3*::GFP)]]. All strains were generously shared by the laboratory of Dr. S.S. Lee (Cornell University, Ithaca, NY). Animals were maintained at 20^{o}C on petri dishes containing nematode growth medium (NGM) seeded with live *E. coli* strain OP50 as the food source according to

the general procedures outlined by Brenner (137).

3.2.4 Lifespan assays

Several gravid adult nematodes were placed on NGM plates seeded with *E. coli* strain OP50 and allowed to lay eggs at 20ºC for approximately 6 hours to obtain a synchronous population. After 6 hours, the nematodes were removed and the plates were placed back at 20ºC until the progeny reached young adulthood (about 72 hours). On day 0 of the experiment, these young adult nematodes were transferred to 35 mm NGM petri dishes containing either no apple phytochemical extracts or the appropriate doses of dissolved apple phytochemical extracts and 50 μM of 5-fluoro-2-deoxyuridine (FUDR) to prevent progeny production. Plates were then dried in a sterile hood, seeded with 100 μL of 3-fold concentrated, saturated *E. coli* OP50 culture, and dried again. Animals were transferred every other day to fresh extracts or control plates until day 8 of adulthood. Lifespan of mutant strains was always conducted in parallel with the wild-type N2 strain to verify the potency of the extracts and to compare the magnitude of the differences between mutant and wild-type animals. Animals were scored daily or every other day by gentle prodding with a platinum wire. Those animals that failed to move were scored as dead. Animals, which exhibited bagging, exploded or crawled off the plates, were censored. Statistical analyses were performed using SPSS statistical software Kaplan-Meier Survival function; *p*-values were obtained using the log-rank test. Unless otherwise indicated, each lifespan experiment was repeated 2-3 times and a representative trial is shown. Unless otherwise indicated, all experiments were performed at 20ºC.

3.2.5 4-HNE assay

4-HNE was measured according to the method described by Loer *et al.* (161) and Wilson *et al.* (108). Briefly, animals were washed off petri plates with M9 buffer, collected into eppendorf tubes and then washed free of bacteria with M9. The supernatant was removed and 500 µL of 4% paraformaldehyde was added. Animals (20-30 per tube) were fixed overnight at 4°C. The following day, animals were washed 3 times in 1 mL of 0.5% TX-100/PBS and incubated at 37°C overnight in 500 µL of 5% BME/1%TX100/0.1M Tris, pH 7.4, with gentle rocking. The following day, animals were washed twice with 1mL of 1% TX-100/0.1M Tris, pH 7.4, washed once with 1mL collagenase buffer (1mM $CaCl_2$, 1% Triton X-100, 0.1M Tris, pH 7.4), and incubated for 45 minutes with 2000 units/mL of Collagenase Type IV. Then, the animals were washed 3 times in 1mL 0.5% TX-100/PBS and blocked for 1 hour in 200 µL of 1% BSA/0.5% TX-100/PBS (AbA) at 4°C. Following blocking, animals were incubated with 100 µl Anti-4-HNE primary antibody (1:100 dilution) in AbA for 2 hours at room temperature, washed 2 times in 1 ml 0.5% TX-100/PBS and incubated overnight with 100 µL Alexaflour 546 goat-anti-mouse secondary antibody (1:100 dilution) in 0.1% BSA/0.5% TX-100/PBS (AbB) at 4°C. The following day, animals were wash 3 times in 1 mL of 0.5% TX-100/PBS and washed once in 1 mL of AbB and left to destain at 4°C in AbB for 24-48 hours. After destaining, individual nematodes were mounted onto 2% agarose slides for fluorescence photomicrography. Slides were visualized with the DM 5000 Leica Microscope using the appropriate filter set. Images were captured using a Hamamatsu ORCA-ER camera with OpenLab software. Quantification of immunofluorescence intensity was done densitometrically by tracing around each animal's pharynx terminal bulb and determining average pixel intensity

using imageJ freeware (NIH) (162).

3.2.6 Stress response induction and GFP visualization

Synchronous populations of CL2166 and CF1553 animals were obtained using the egg lay method as described above. Upon reaching young adulthood GFP animals were placed on petri dishes containing either no or appropriate amounts of apple phytochemical extracts and maintained at 20°C. Following 48 hours of treatment, animals were anesthetized in 20 µM sodium azide and mounted on 2% agarose slides for visualization. For the CL2070 strain, animals were treated with apple phytochemical extracts for 48 hours starting from young adulthood, heat-induced at 35°C for 2 hours and returned to 20°C to recover. 20 hours following heat induction, animals were anesthetized and mounted on slides as above. Slides were visualized using the Leica DM5000B Microscope (Bannockburn, IL), with the appropriate GFP filter. GFP fluorescence was captured using a Hamamatsu ORCA-ER camera with the OpenLab software. Using ImageJ freeware (NIH) (162), the fluorescence intensity of 20-30 animals per group was measured as the sum intensity of all pixels within a 75-255 threshold in 8-bit grayscale images containing a single worm each.

3.2.7 Statistical analyses

Survival data were analyzed using SPSS version 16 for Windows (SPSS Inc., Chicago, IL) Kaplan-Meier Survival function, Kaplan-Meier Estimator of means, and log-rank test. All other analyses were done using Minitab statistical software (State College, PA). A p- value < 0.05 was considered to be statistically significant among treatments in multiple

comparisons for all statistical tests performed. Data for 4-HNE and stress response induction were analyzed using One-way ANOVA (assuming equal variance), with Tukey's multiple comparison test for differences among groups where appropriate. A *p*- value < 0.05 was considered to be statistically significant Graphs were plotted with the SigmaPlot version 10 for Windows software (Systat Software Inc., San Jose, CA).

3.3 Results

3.3.1 Effect of apple phytochemicals on insulin signaling, sirtuin and mitochondrial respiration mutants

In *C. elegans* the modulation of insulin signaling, sirtuins, and oxidative stress have been shown to affect lifespan. To determine if apple extracts extend lifespan by any of these pathways, we used mutant strains of *daf-16(mgDf47), sir2.1(ok434)* and *mev-1(kn1)* to perform lifespan analyses in parallel with wild-type N2 animals. *daf-16* is a transcription factor which is activated under conditions of stress such as starvation, overcrowding or elevated oxidation. It is regulated by insulin signaling and binds to the promoters of many genes responsible for stress response, metabolism, fat storage and lifespan. *daf-16* mutants are short-lived. *sir-2.1* belongs to the silent information regulator (SIR) family of proteins known as the sirtuins, which are conserved in yeast, worms, flies and mammals. The sirtuins are part of a family of NAD+-dependent deacetylases that play an important role in gene silencing, DNA repair and aging in a diverse group of organisms (89). In *C. elegans*, *sir-2.1* overexpression leads to long life, this effect is dependent on *daf-16* (91). *mev-1* is an mitochondrial mutant with a mutation in the cytochrome *b* subunit of complex II. It is hypersensitive to oxidative stress and

short-lived.

The mean lifespan of N2 animals was 17.01±.30 days. After administering 5, 10 and 20 mg/mL of apple phytochemical extracts, the mean lifespan increased to 23.81±.65, 22.26±.44 and 20.72±.36 days, respectively. The dose of 5 mg/mL produced the highest increase in lifespan of 40% over the control (Table 3.1). Likewise, *daf-16* animals lived an average of 15.80±.25, 19.08±.30, 19.38±.30 and 19.80±.40 days for 0, 5, 10 and 20mg/mL groups, respectively. This represents an increase of up to 25.3% in a dose-dependent manner over the 0 mg/mL *daf-16* control (Table 3.1). Similarly, *sir2.1* animals survived for 17.65±.29, 22.75±.43, 21.82±.43, 20.76±.36 days in 0, 5, 10 and 20 mg/mL groups. The 5 mg/mL dose yielded the highest increase in lifespan of up to 28.9% over the 0 mg/mL *sir2.1* control (Table 1). Finally, the lifespan of *mev-1* animals was 14.63±.50, 16.57±.47, 16.78±.50, 17.75±.52 days for 0, 5, 10 and 20 mg/mL groups, respectively. This represents an increase of up to 21.3% in a dose-dependent manner over the 0 mg/mL *mev-1* control (Table 3.1). It is of note that many lifespan experiments showed maximum benefit at lower doses.

3.3.2 Effect of apple phytochemicals on calorie restricted animals

We examined the effect of apple phytochemical extracts on the lifespan of the calorie restricted *eat-2* animals. These animals have a pumping defect, which causes them to pump less frequently and thus calorie restrict themselves. *eat-2* worms are long-lived compared to the wild-type. The lifespan of animals on 0, 5, 10 and 20 mg/mL of apple phytochemical extracts was 22.30±.51, 31.85±.83, 33.53±.63, 27.03±.52 days, respectively. The dose of 10 mg/mL showed the highest increase in lifespan of 50.4% over the *eat-2*

control (Table 3.1). In parallel, we performed N2 lifespan and found that mean lifespan was 16.08±.28, 24.35±.49, 25.58±.70, 25.09±.68 days for 0, 5, 10 and 20 mg/mL, respectively. The dose of 10 mg/mL showed the highest increase in lifespan of 59.1% over the control (Table 3.1).

3.3.3 Effect of apple phytochemicals on stress response induction

The induction of superoxide dismutase, glutathione-S-transferase and the small heat shock protein promoter *hsp16.2* was examined using a GFP reporter system. Apple phytochemical extracts significantly decreased the activity of the *sod-3* reporter in all treatment groups versus the 0 mg/mL control by up to 70% (Figure 3a; $p<0.01$, One-way ANOVA). Likewise, apple phytochemical extracts decreased the activity of the *gst-4* promoter in 5 and 10 mg/mL groups versus the 0 mg/mL control by up to 35% (Figure 3b; $p<0.01$, One-way ANOVA). In contrast, apple phytochemical extracts increased the activity of the *hsp16.2*::promoter by up to 30% versus the 0 mg/mL control (Figure 3c; $p<0.01$, One-way ANOVA).

Table 3.1 Effect of apple phytochemical extracts on mean lifespan of wild-type and mutant *C. elegans* nematodes.

Genotype	Treatment (mg/mL)	N	Mean Lifespan±SEM (days)*	Δ**	*p*-value vs. control	% of Control
N2 (wild-type) parallel to *daf-16, sir2.1, mev-1*	0 (control)	80	17.01±.30	a	N.A.	100.0
	5	74	23.81±.65b	b	<0.001	140.0
	10	91	22.26±.44	c	<0.001	130.9
	20	85	20.72±.36	d	<0.001	121.8
N2 parallel to *eat-2*	0	55	16.08±.28	a	N.A.	100.0
	5	80	24.35±.49	b	<0.001	151.4
	10	81	25.58±.70	b	<0.001	159.1
	20	79	25.09±.68	b	<0.001	156.0
daf-16 (*mgDf47*)	0	87	15.80±.25	a	N.A.	100.0
	5	90	19.08±.30	b	<0.001	120.8
	10	93	19.38±.30	b	<0.001	122.7
	20	79	19.80±.40	b	<0.001	125.3
sir2.1(ok434)	0	72	17.65±.29	a	N.A.	100.0
	5	97	22.75±.43	b	<0.001	128.9
	10	82	21.82±.43	b	<0.001	123.6
	20	78	20.76±.36	c	<0.001	117.6
eat-2(ad465)	0	73	22.30±.51	a	N.A.	100.0
	5	75	31.85±.83	b	<0.001	142.8
	10	68	33.53±.63	b	<0.001	150.4
	20	59	27.03±.52	c	<0.001	121.2
mev-1(kn1)	0	69	14.63±.50	a	N.A.	100.0
	5	68	16.57±.47	b	0.016	113.3
	10	68	16.78±.50	b	<0.01	114.7
	20	77	17.75±.52	b	<0.001	121.3

Table 3.1 (Continued)

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

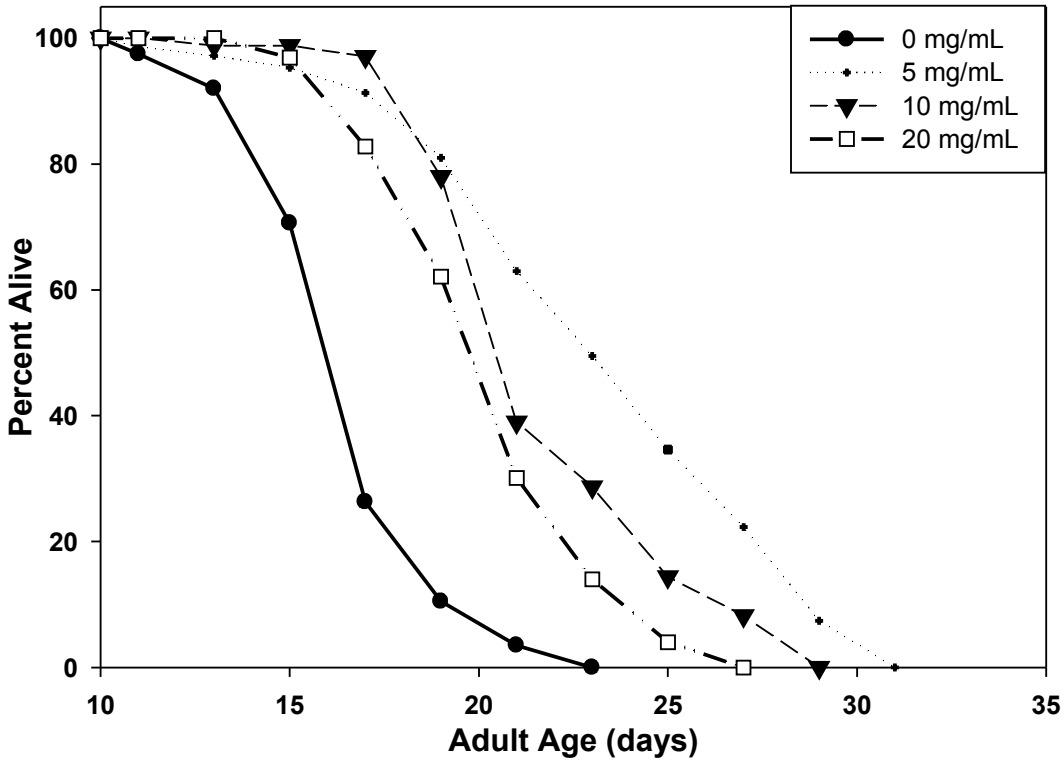


Figure 3.1 Effect of apple phytochemical extracts on adult lifespan of N2 wild-type animals done in parallel with *daf-16(mgDf47)*, *sir2.1(ok434)* and *mev-1(kn1)* mutant animals (see figures below). N2 day 1 young adult wild-type N2 animals were treated with the indicated concentrations of apple extracts or no extract and monitored for survival starting on the first day of adulthood. Animals that were exposed to three levels of apple extracts survived significantly longer than those that were not (p<0.001 for 0 mg/mL compared to each treatment group; log-rank test; see Table 3.1). The experiment was repeated multiple times and a representative trial is shown.

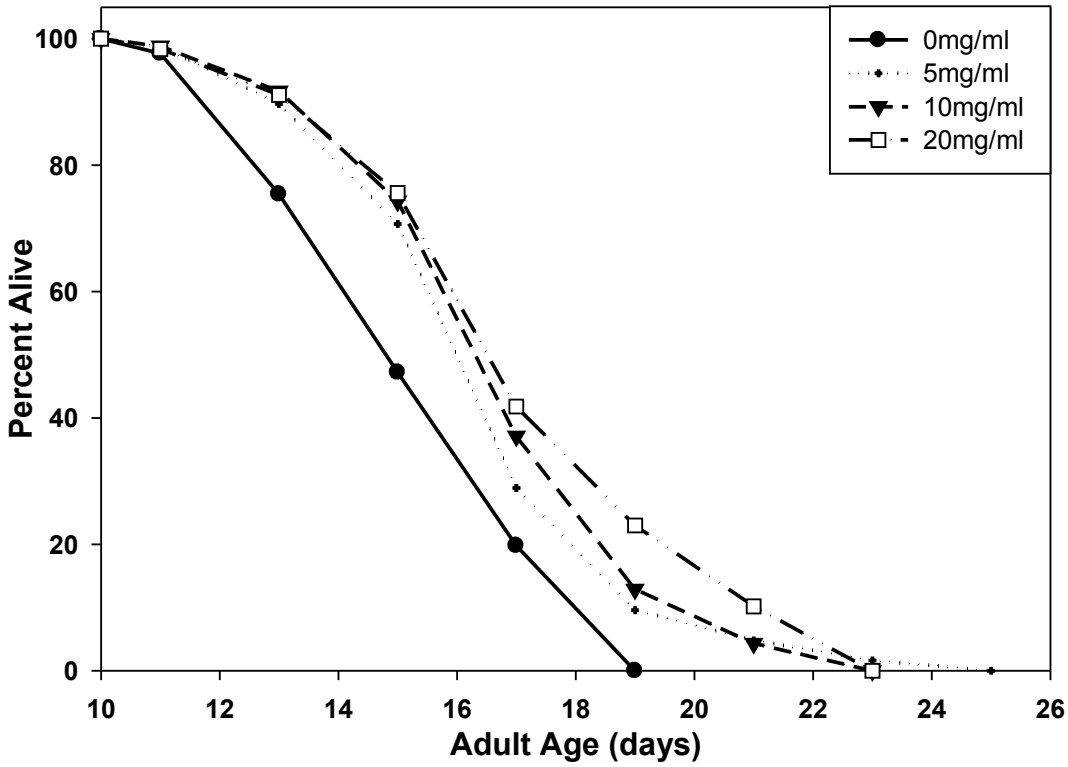


Figure 3.2 Effect of apple phytochemical extracts on adult lifespan of *daf-16(mgDf47)* animals. Day 1 young adult *daf-16(mgDf47)* animals were treated with the indicated concentrations of apple extracts or no extract and monitored for survival starting on the first day of adulthood. Animals that were exposed to three levels of apple extracts survived significantly longer than those that were not (p<0.001 for 0 mg/mL compared to each treatment group; log-rank test; see Table 3.1). The experiment was repeated multiple times and a representative trial is shown.

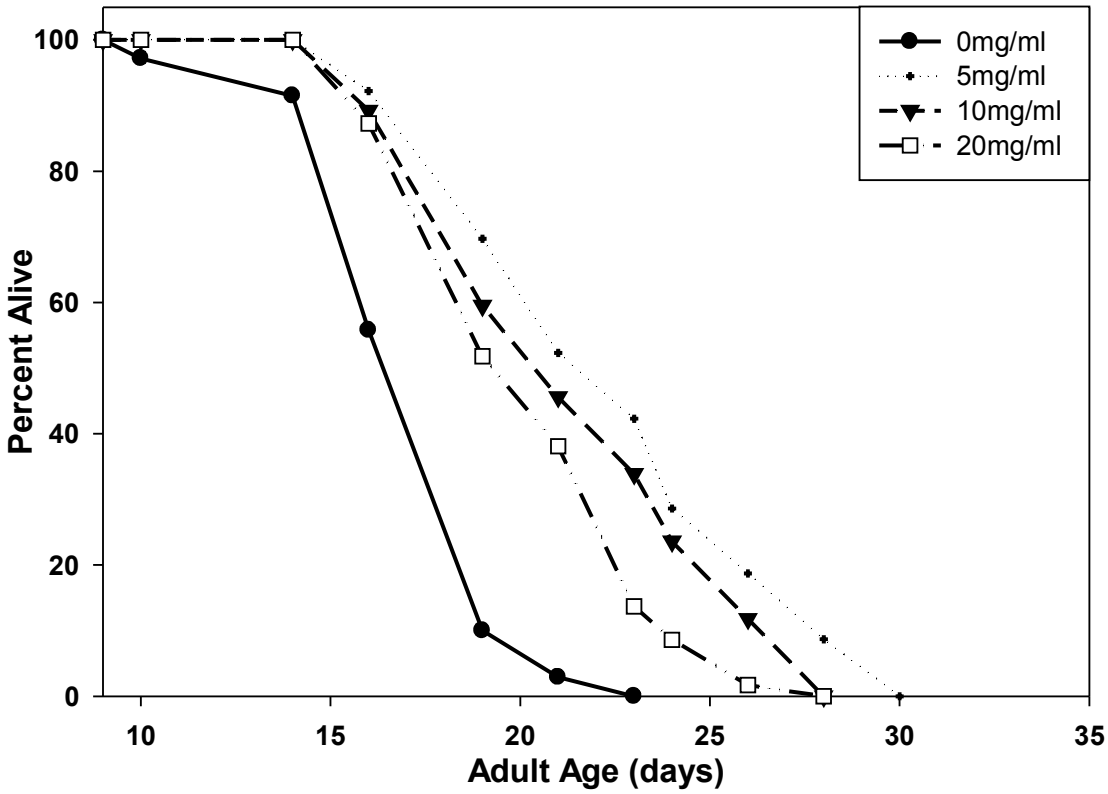


Figure 3.3 Effect of apple phytochemical extracts on adult lifespan of *sir2.1(ok434)* animals. Day 1 young adult *sir2.1(ok434)* animals were treated with the indicated concentrations of apple extracts or no extract and monitored for survival starting on the first day of adulthood. Animals that were exposed to three levels of apple extracts survived significantly longer than those that were not (p<0.001 for 0 mg/mL compared to each treatment group; log-rank test; see Table 3.1). The experiment was repeated multiple times and a representative trial is shown.

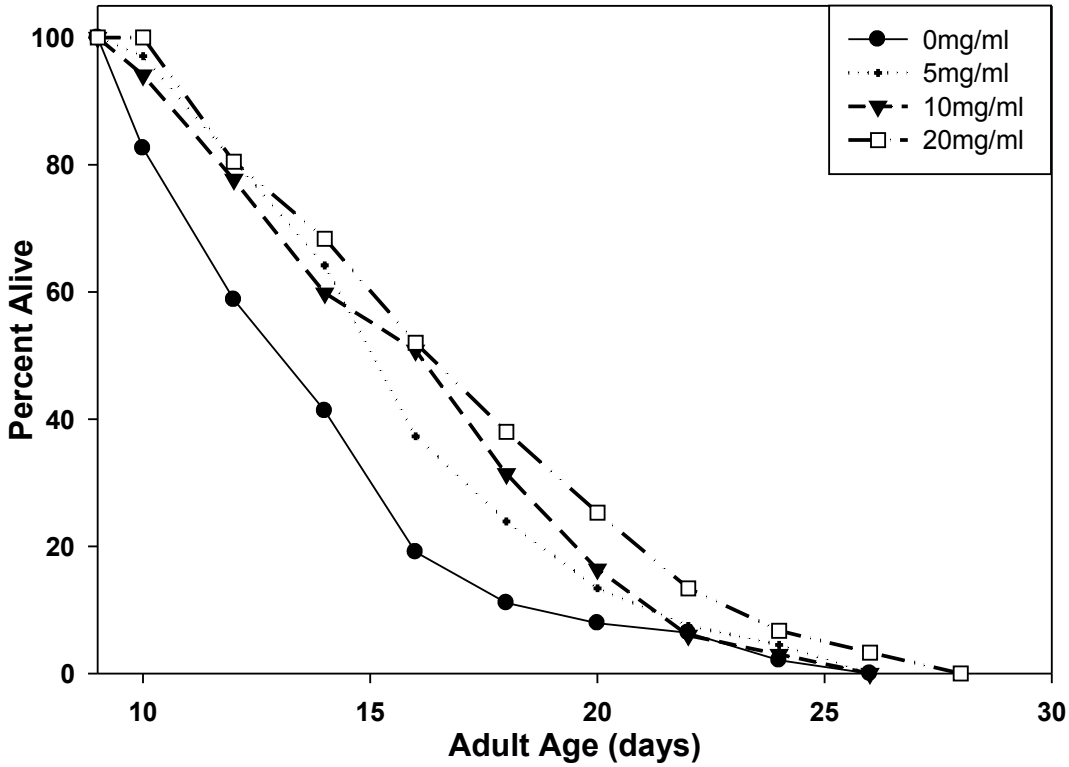


Figure 3.4 Effect of apple phytochemical extracts on adult lifespan of *mev-1(kn1)* animals. Day 1 young adult *mev-1(kn1)* animals were treated with the indicated concentrations of apple extracts or no extract and monitored for survival starting on the first day of adulthood. Animals that were exposed to three levels of apple extracts survived significantly longer than those that were not (p<0.05 for 0 mg/mL compared to each treatment group; log-rank test; see Table 3.1). The experiment was repeated multiple times and a representative trial is shown.

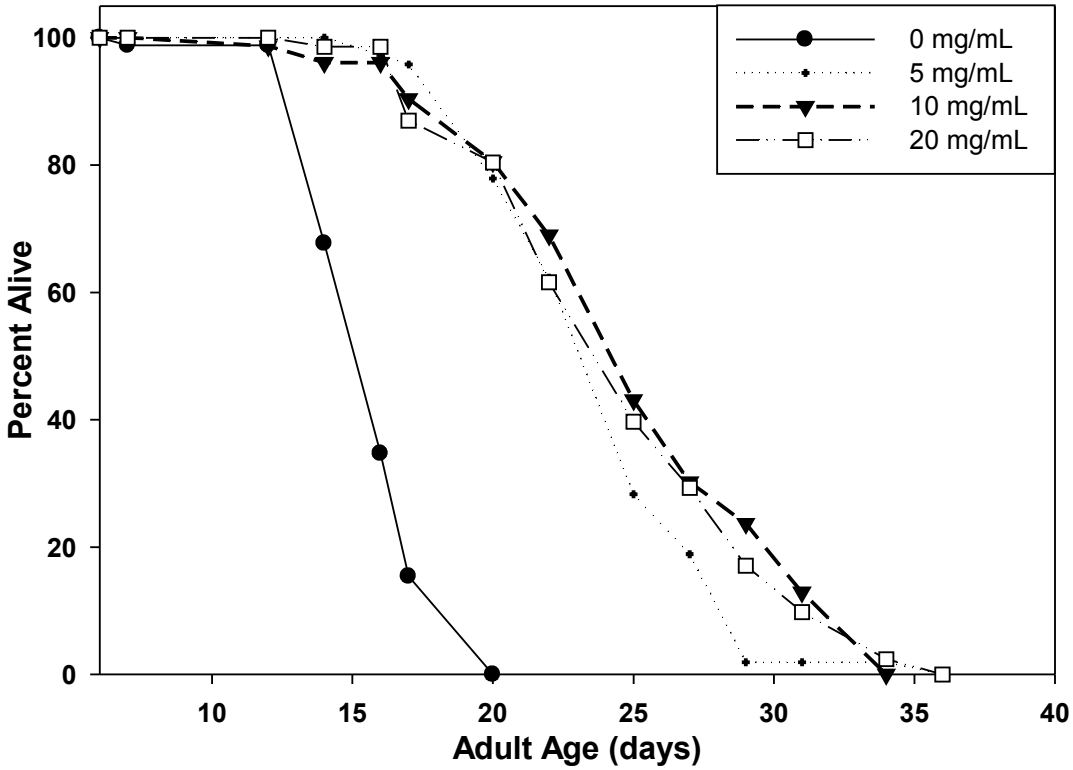


Figure 3.5 Effect of apple phytochemical extracts on adult lifespan of N2 animals done in parallel to *eat-2(ad465)*. Day 1 young adult N2 animals were treated with the indicated concentrations of apple extracts or no extract and monitored for survival starting on the first day of adulthood. Animals that were exposed to three levels of apple extracts survived significantly longer than those that were not (p<0.001 for 0 mg/mL compared to each treatment group; log-rank test; see Table 3.1). The experiment was repeated multiple times and a representative trial is shown.

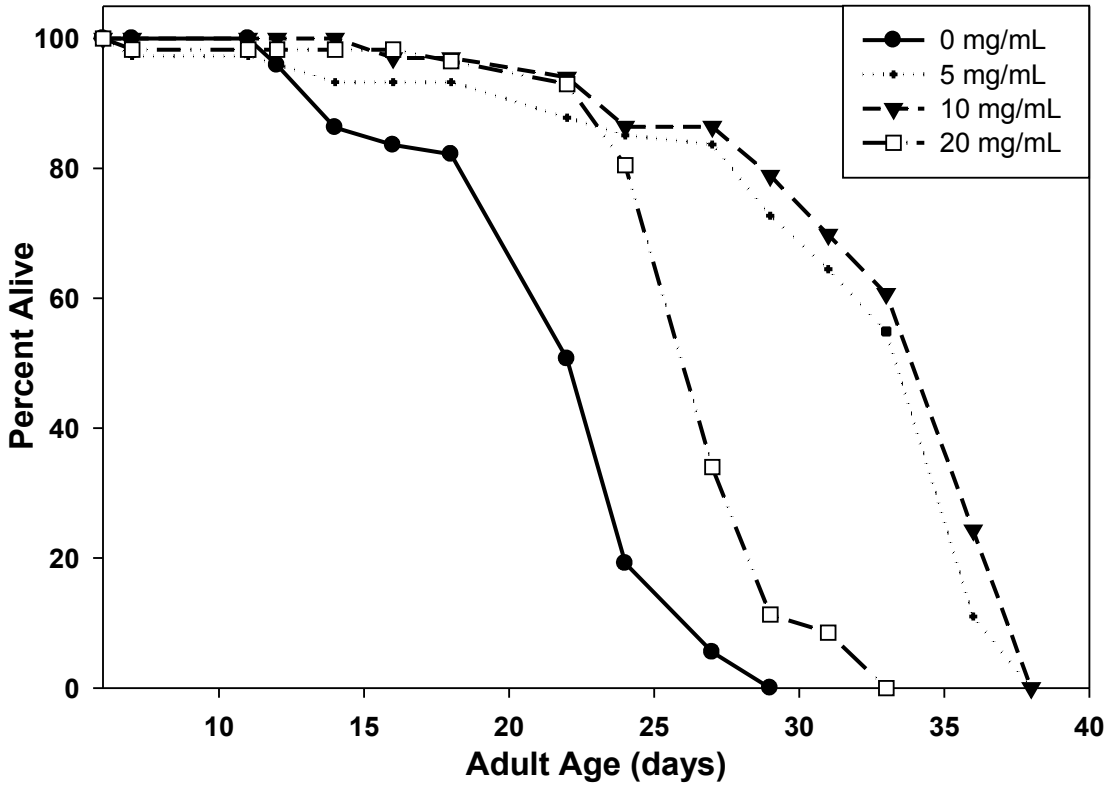
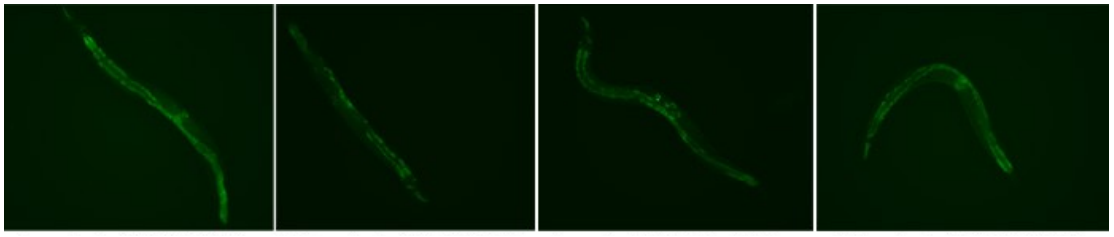


Figure 3.6 Effect of apple phytochemical extracts on adult lifespan of *eat-2(ad465)* animals. Day 1 young adult *eat-2(ad465)* animals were treated with the indicated concentrations of apple extracts or no extract and monitored for survival starting on the first day of adulthood. Animals that were exposed to three levels of apple extracts survived significantly longer than those that were not (p<0.001 for 0 mg/mL compared to each treatment group; log-rank test; see Table 3.1). The experiment was repeated multiple times and a representative trial is shown.



0 mg/mL SOD-3::GFP 2.5 mg/mL SOD::GFP 5 mg/mL SOD::GFP 10 mg/mL::SOD-3::GFP

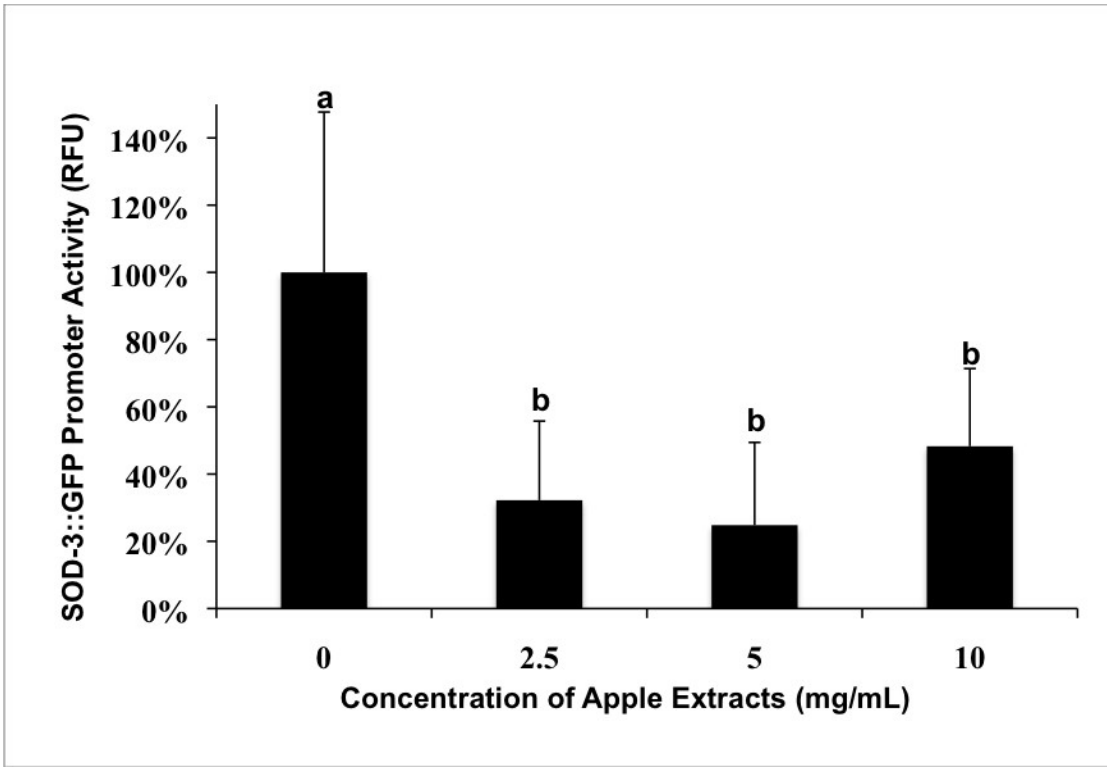


Figure 3.7 Effect of apple phytochemical extracts on SOD-3::GFP promoter activity. N=23, 19, 4 and 22 animals for 0, 2.5, 5 and 10 mg/mL groups, respectively; $p<0.01$ for 0 mg/mL versus each apple extract group; One-way ANOVA, Tukey's multiple comparisons; all other comparisons not significant). Bars with no letters in common represent groups that are statistically significantly different. Values are expressed as mean relative fluorescence ± SD. See Appendix II for additional statistical analysis.

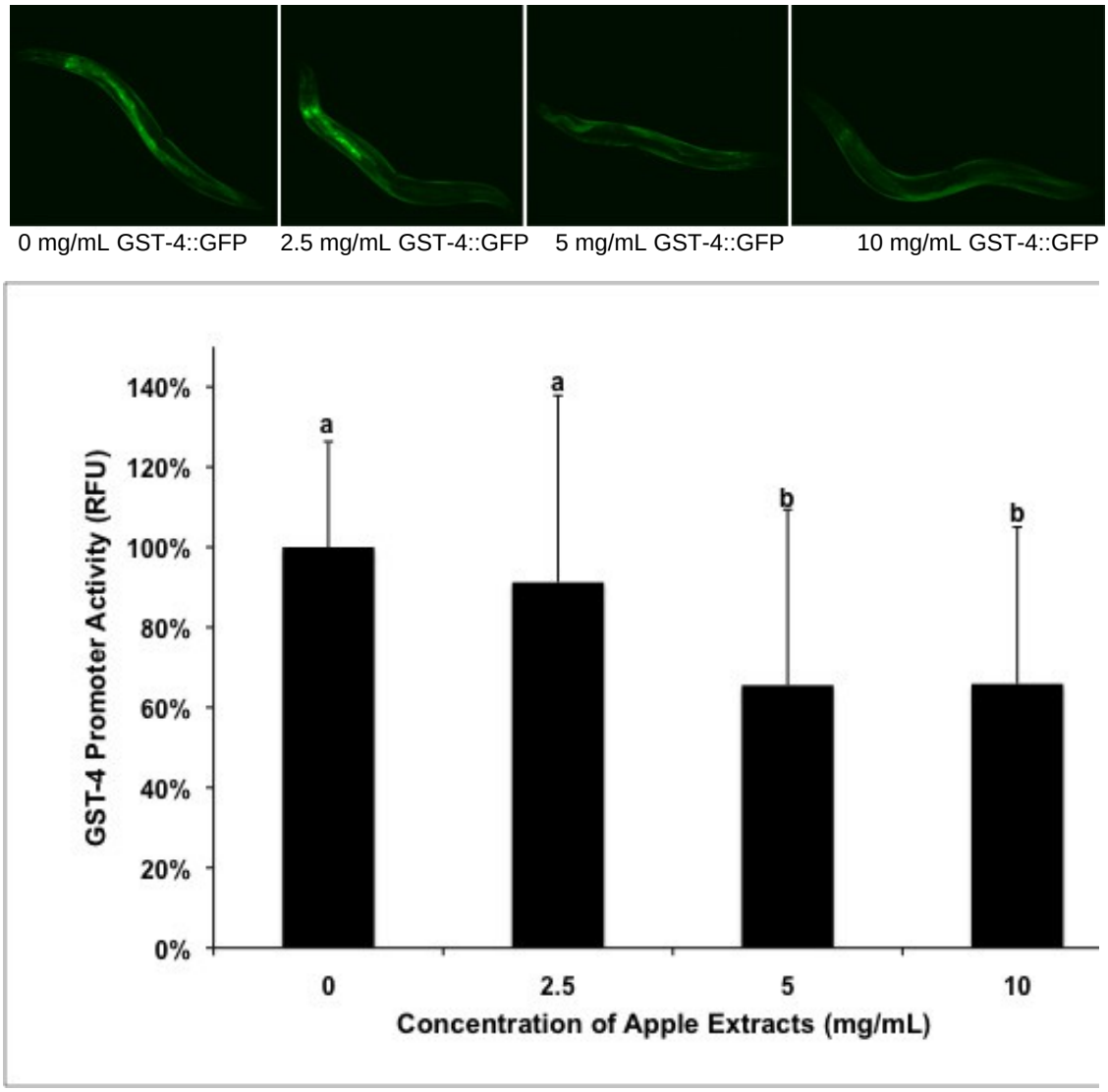


Figure 3.8 Effect of apple phytochemical extracts on GST-4 promoter activity (N = 33, 28, 28 and 27 animals for 0, 2.5, 5 and 10 mg/mL groups, respectively; $p<0.01$ for 0 mg/mL vs. 5 mg/mL and for 0 mg/mL vs. 10mg/mL groups; $p=0.044$ for 2.5 mg/mL vs. 5 mg/mL groups; One-way ANOVA, Tukey's multiple comparisons). Bars with no letters in common represent groups that are statistically significantly different. Values are expressed as mean relative fluorescence ± SD. See Appendix II for additional statistical analysis.

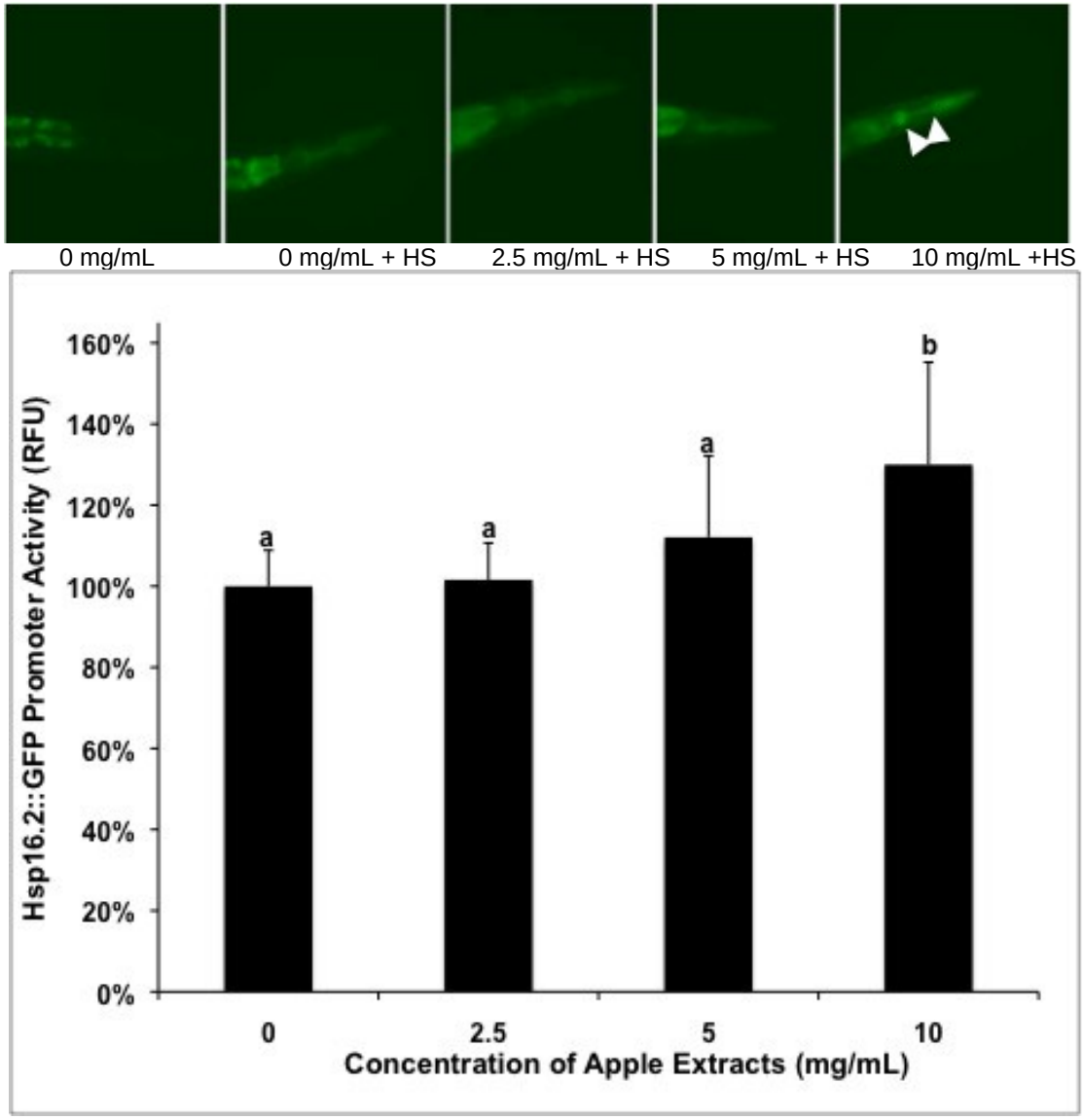


Figure 3.9 Effect of apple phytochemical extracts on Hsp16.2 promoter activity (*n*=24, 23, 27 and 20 animals for 0, 2.5, 5 and 10 mg/mL groups, respectively; $p<0.01$ for 0 vs. 10 mg/mL, One-way ANOVA, Tukey's multiple comparisons; all others nonsignificant). Bars with no letters in common represent groups that are statistically significantly different. Values were expressed as mean relative fluorescence ± SD. See Appendix II for additional statistical analysis.

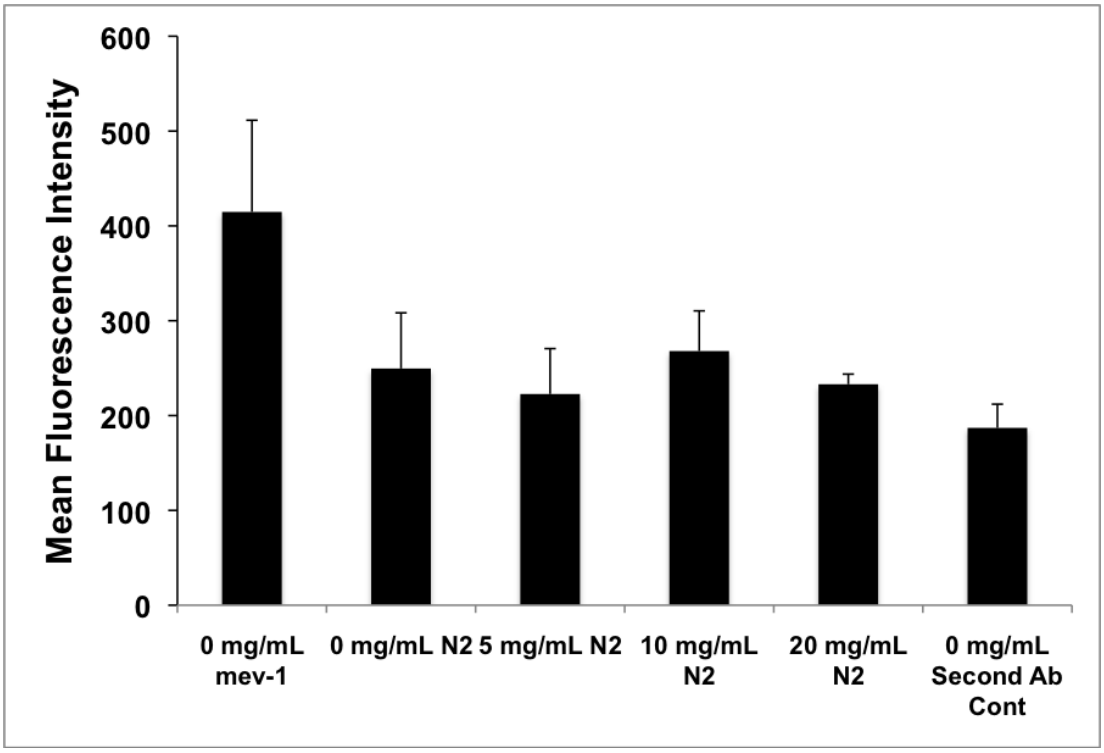


Figure 3.10 Effect of apple phytochemical extracts on 4-HNE accumulation in *C. elegans*. N≤10 animals per group. There were no significant differences in the accumulation of 4-HNE lipid peroxidation byproduct. (p=.193 0 mg/mL versus all treatment groups; One-way ANOVA and p<0.001 0 mg/mL N2 versus 0 mg/mL *mev-1(kn1)* positive control; One-way ANOVA; Tukey's multiple comparisons). A p-value < 0.05 was considered to be significantly different. See Appendix II for additional statistical analysis.

3.4 Discussion

This study sought to characterize the enhanced lifespan and stress response mechanisms of apple phytochemical extracts using the *C. elegans* model. We performed a genetic screen of mutant animals lacking genes in the five major longevity pathways. We reasoned that lifespan extension with apple phytochemical extracts would be abrogated in animals lacking a gene in a pathway that is required for this effect. Our results indicate that, in *C. elegans*, lifespan extension with apple phytochemical extracts is most likely not due to insulin signaling, sirtuin activity, signaling from the germ line or calorie restriction. While the increase in lifespan of *daf-16* and *sir2.1* mutant strains we observed was somewhat smaller than that of N2 animals, we believe that these differences were within the acceptable range of variation between experiments. However, we cannot exclude the possibilities that these differences are due to differential metabolism of the compounds, deleterious pleiotropic interactions between the mutation and phytochemicals, or other physiological differences between the wild-type and mutant animals (112). Thus, the possibility remains that these pathways may be at least partially responsible for mediating the effects of apple phytochemical extracts. Interestingly, previous reports have shown that other dietary phytochemicals such as resveratrol, a stilbene found in grapes, peanuts and other plant foods, and quercetin, a flavonoid found predominantly in apples and onions, exert their effects via modulating sirtuin and insulin signaling, respectively. Our findings suggest that apple phytochemicals do not act mainly via the major longevity pathways, but instead have a unique mode of action owing to the variety of compounds they contain.

Apple phytochemical extracts have potent antioxidant activity *in vitro* (39,

49). Previously, we showed that apple phytochemical extracts also have antioxidant effects *in vivo* in a paraquat-induced model of oxidative stress. To further characterize the possible antioxidant effects of apple phytochemical extracts in *C. elegans*, we used the *mev-1(kn1)* strain, which has a missense mutation in the *cyt-1* gene coding for the succinate dehydrogenase cytochrome *b* large subunit of complex II. The animals in this strain are hypersentive to oxygen, produce excessive amounts of superoxide anions in their mitochondria and have a shortened lifespan. In addition, they also have lower superoxide dismutase levels, reduced glutathione levels, and higher lactate concentrations (96, 163, 164). Our results show that *mev-1* animals treated with apple phytochemical extracts had an increase in lifespan similar to that of wild-type animals. We interpret this result to mean that apple phytochemical extracts alleviated damage caused by oxidative stress, in particular superoxide anions, and thereby improved the survival of *mev-1* animals. This biochemical effect is separate from the genetic effect of the *cyt-1* gene, which plays a key role in energy metabolism and superoxide anion production (163).

Damage from oxidative stress can affect DNA, proteins, lipids, and other macromolecules. To further investigate the type of oxidative damage that may be attenuated by apple phytochemical extracts, we examined levels of 4-Hydroxy-2-Nonenal (4-HNE) in wild-type control and wild-type apple phytochemical extracts treated animals. 4-HNE is a byproduct of lipid peroxidation, which results from ROS peroxidation damage to polyunsaturated fatty acids. It has been shown to accumulate with age in many species. In *C. elegans* increasing the rate of metabolism of 4-HNE increases lifespan (71, 165). Our results did not show a significant effect of apple phytochemical

extracts on the amount of 4-HNE relative to the control (Figure 4c). Interestingly, Wilson *et al.* have reported that in *C. elegans*, blueberry polyphenols decrease the levels of 4-HNE, showing that at least some dietary polyphenols can attenuate 4-HNE in this model (108).

Increased lifespan often correlates with increased stress resistance (120). Previously, we showed that survival upon challenge with oxidative stress, UV irradiation, pathogenic infection and heat stress were all improved following treatment with apple phytochemical extracts. To further characterize the ability of animals to cope with stress, we examined the expression of genes that are associated with oxidative stress response by looking at GFP expression of *sod-3* and *gst-4* reporter strains. These strains contain GFP driven by the superoxide dismutase (*sod-3*) and glutathione-S-transferase *(gst-4)* promoters, respectively. Since both *sod-3* and *gst-4* are induced by oxidative stress, we used the expression of GFP as a proxy of ROS damage.

sod-3 is one of five isoforms of superoxide dismutase found in *C. elegans*. It is expressed in the mitochondrial matrix where it quenches superoxide anions. The other superoxide dismutases are the mitochondrial MnSOD *sod-2*, two cytosolic Cu/Zn isoforms: *sod-1* and *sod-5*, and an extracellular Cu/ZnSOD, *sod-4*. SOD-3::GFP localizes predominantly to the pharynx and intestine. Honda *et al.* have shown that deletion of the two MnSODs causes increased sensitivity to paraquat and hyperoxia, but has no effect on lifespan, or even increases it in some cases (166). Similarly, others have shown that deleting all five *sod* enzymes increases oxidative stress, but does not shorten lifespan, and even lengthens it in some cases (67, 68). However, in long-lived insulin signaling *daf-2* mutants, *daf-16* translocates into the nucleus where it directly binds the SOD-3 promoter and upregulates the

SOD-3 gene. While this effect appears not to be directly causal of enhanced lifespan (68), *sod-3* still reduces oxidative damage and can thereby promote health and lifespan indirectly.

In our experiments, apple phytochemical extracts reduced the activity of the *sod-3* promoter. We propose that apple phytochemical extracts may protect against deleterious oxidative damage normally mitigated by *sod-3* and thus lead to a decreased need for this part of the endogenous antioxidant system. In agreement with this, we previously found that healthspan of our animals, as indicated by lipofuscin attenuation and motility, was also significantly improved. Since we did not address the functional interaction between the apple phytochemical extracts and *sod-3* directly, the possibility exists that apple phytochemical extracts may act by repressing this antioxidant gene directly rather than by attenuating oxidative damage. However, taken together with our *mev-1* survival and previous paraquat results the latter seems to be the more likely possibility.

Similarly, results with *gst-4::*GFP showed that the induction of this transgene was reduced upon exposure to apple phytochemical extracts. *gst-4* is a glutathione-S-transferase which functions in the detoxification of endogenous OS products and xenobiotics.

We propose that, antioxidant compounds from apple phytochemical extracts act to mitigate oxidative damage, thus reducing the need for the activity of *gst-4*. As with *sod-3,* however, functional effects cannot be ruled out.

In a previous experiment, we found that thermotolerance was improved upon treatment with apple phytochemical extracts. Heat shock response is a highly conserved process that functions to maintain cellular homeostasis. Therefore, we examined the small heat shock protein *hsp16.2,* which is

upregulated under a variety of stress conditions including thermotolerance, oxidative stress and detoxification of some environmental toxins. *hsp16.2* has also been shown to interact with intracellular human beta amyloid peptide, likely as a stabilizing chaperone protein, making it of interest in studies involving models of Alzheimer's disease.

Hsp-16.2::GFP expression is strongest in the intestine and the pharynx (167). Because *hsp16.2* is induced only under conditions of stress, especially heat and oxidative stress, we exposed the animals to heat shock prior to visualizing GFP activity. After treating the *hsp16.2*::GFP animals with apple phytochemical extracts, we observed an induction of the *hsp-16.2* promoter. We propose that under conditions of stress, apple phytochemical extracts produce a heat stress response via *hsp16.2*, a protein that can mitigate byproducts of oxidative and environmental stresses, and endogenous and exogenous toxins, as well as stabilize misfolded proteins.This idea is consistent with our previous findings that apple phytochemical extracts treated animals survive longer following heat stress than do controls animals. The induction of *hsp16.2* under conditions of stress may also explain the improved response to other stressors that were tested such as paraquat-induced oxidative stress and UV irradiation. This idea is in agreement with previously published reports, which found that plant extracts, including those of plant adaptogens and *Ginko biloba* upregulated *hsp16.2* in the *C. elegans* model (109, 168).

3.5 Conclusion

We propose that the activity of apple phytochemical extracts is not dependent on the insulin signaling pathway, *sir2.1*, or calorie restriction.

Instead, the effects appear to be mediated by attenuation of ROS damage. This view is supported by the increased lifespan of the *mev-1* mutant, increased survival upon induction of paraquat-induced oxidative stress and a reduction of induction of the *sod-3* and *gst-4* GFP promoters. However, because we saw no effect on the attenuation of 4-HNE, we cannot definitively conclude that apple phytochemical extracts act to mitigate oxidative damage at the macromolecular level. Further evidence such as protein and DNA oxidation data, would be needed before such a conclusion could be reached. In addition, increased induction of *hsp16.2* upon conditions of stress likely contributes to a general improvement of cellular detoxification and stress response, and explains improved survival under conditions of heat shock, oxidation stress, and UV irradiation. Our findings are in agreement with the 'green theory of aging' which posits that molecular junk, including oxidation byproducts, accumulates with age and, if left undetoxified by phase I and II systems, leads to the molecular damage associated with aging. In addition, a recent report by Benedetti *et al.* showed that, out of a wide spectrum of antioxidants, only a select few extend lifespan of *C. elegans.* Of these, one compound showed an induction of CYP35::GFP, a phase I detoxification gene associated with the xenobiotic response (121). Further studies into the potential type(s) of oxidative damage that may be reduced, as well as other potential mechanisms of action that may be involved would be of great interest. Additionally, it would also be interesting to elucidate the individual combinations of phytochemicals that are responsible for increasing lifespan and improving stress response.

3.6 Acknowledgements

We thank Judy Liang and Carolle Mok for technical assistance. We thank Atsushi Ebata, Nicole Liachko, Gizem Rizki and Ludivine Walter for helpful suggestions and experimental troubleshooting. We are grateful to Mark Wilson for sharing his 4-HNE protocol and Jason Neuswanger for help with statistical analysis and editing of the manuscript.

CHAPTER 4

Conclusions and future perspectives

Aging research has made tremendous strides in the last 20-30 years. It was shown that the aging process is plastic, and that this plasticity can be manipulated by altering genes, the physical environment and diet. This latter manipulation in particular has seen a great deal of interest and surge in publications in recent years, especially in the model *C. elegans*. In this model, a variety of treatments including vitamins, clinical drugs, synthetic antioxidants and phytochemicals, have shown that it is possible to not only extend lifespan but also reduce age-related decline and pathologies such as sarcopenia and accumulation of human beta amyloid peptiedes which are associated with Alzheimer's disease. *C. elegans* is one of the most popular and robust, simplest whole-animal model systems that has been employed in the study of aging. Results from *C. elegans* taken together with those of other organisms such as *Saccharomyces cerevisiae, D. melanogaster, Mus musculus* and primates, can provide a powerful comparative approach in the study of nutrition, natural products and lifespan.

Apples are the second most highly consumed fruit and the number one source of phenolics in the United States. Apple phytochemicals have been shown to have a wide variety of health benefits including antioxidation, antiproliferation and modulation of a variety of molecular targets such as NF-kB and AP-1. The work presented here shows for the first time that apple phytochemicals can increase lifespan and healthspan *in vivo* using *C. elegans*. This increase appears to be dose-dependent, at least some of the time and is accompanied by an improvement in motility and attenuation of lipofuscin. In addition, I have shown that apple phytochemicals increase

resistance to various stresses such as oxidative, heat, UV, and pathogenic infection. The mechanism by which apple phytochemicals exert their effects do not appear to be through insulin signaling, sirtuin *sir2.1*, calorie restriction or germ line signaling. Instead, the evidence points to attenuation of oxidative stress and a generalized cellular stress resistance response as the two mechanisms responsible for the observed effects. This conclusion is supported by increased lifespan of the *mev-1* mutant, resistance to paraquat-induced oxidative stress and reduced levels of expression of sod-3::GFP and gst-4::GFP, two enzymes responsible for sttenuating oxidative damage. In addition, induction of the hsp16.2 promoter was increased following heat stress suggesting that there is a protective and possibly generalized increase in the cellular stress response. These two processes likely contribute to the increase in lifespan, healthspan and stress resistance that I observed in *C. elegans* in this study. The examination of additional markers of oxidative stress such as DNA and protein oxidation would be of great interest.

Apples possess a wide variety of compounds, the most bioactive of which are in the flavonoids group. I tested two such compounds, quercetin-β-D-glucoside and 2-α-ursolic acid and found that both increased lifespan of *C. elegans*. However, I did not observe a dose-response and the increase in lifespan was not as great as that for the whole apple phytochemical extracts. This suggests that additive and/or synergistic interactions between different compounds are needed to elicit the maximum effect. It is worth pointing out that the anti-aging activity of apples is unique. At the onset of my research, I examined cranberry extracts and found that there was no effect on aging in *C. elegans* at the doses tested. In addition, only one other (berry) fruit, blueberries, have been shown to extend lifespan. While this study was the first

of its kind and is highly significant, apples are much more highly consumed than blueberries and are thus of more relevance. Additionally, the bioactive compounds of blueberries, that were found to exert the anti-aging effects on *C. elegans*, the proanthocyanidins, have low biovailability in humans (169). Also, a wild blueberry juice preparation was used for fractionation, whereas most blueberries and blueberry juices purchased by consumers are of the commercial blend and likely have different phytochemical profiles.

My findings are significant because while it has been known for a long time that apples are “good for you,” the anti-aging, healthspan, and stress resistance effects of apples and their mechanisms *in vivo* have never been previously published. However, further examination into additional mechanisms of action would be of great interest.

For example, to better characterize the type and extent of oxidative damage, we could use the *skn-1* mutant. This gene is involved in the oxidative stress response of *C. elegans*. SKN-1 is a gene related to the vertebrate Nrf proteins, and a transcription factor that acts in parallel to DAF-16 to promote the expression of phase II detoxification enzymes, especially in response to oxidative stress. SKN-1 is a target of IIS, but acts independently of DAF-16. Like DAF-16, SKN-1 is sequestered in the cytoplasm until it is activated by p38 phosphorylation under conditions of stress. Upon activation, SKN-1 translocates to the nuclei of intestinal cells where it binds to the promoters of and turns on a variety of target genes, which act to reduce oxidative stress. If the lifespan increase of *skn-1* mutants is abrogated with respect to N2, this might suggest that apple phytochemicals act through this transcription factor. If this were the case, we could examine the relative expression of specific targets of SKN-1 such as promoters of *gcs-1*, *gst-4, gst-7, gst-5* and *gst-10*

using RT-PCR. Additionally, if GFP strains of SKN-1 and/or its target genes exist, it would be facile to examine the expression pattern of these reporter strains. It might also be of interest to measure the actual levels of ROS inside the worms and compare these between controls and apple treated animals. Recently, it was reported that electron paramagnetic resonance spectroscopy was used for this purpose in *C. elegans* (170). This technique takes advantage of the correlation between free iron species and free superoxide anions inside the cell. By measuring the amount of iron, it is possible to gauge the levels of superoxide and its damage.

In addition, RT-PCR of *sod-3* and *gst-4* could be carried out to confirm the GFP results of the reduced activity of these two genes. Moreover, it would be worthwhile to examine the other SOD genes (there are a total of five in *C. elegans*) and other GST genes to see if they are also involved in reducing oxidative damage upon treatment with apple phytochemical extracts and to what extent.

Other potential experiments include using DAF-16::GFP to test whether this transcription factor is activated and gets into the nucleus. While my lifespan data suggest that this is probably not the case, the increase in lifespan that was observed was smaller than that of N2 animals leaving the possibility that DAF-16 plays some role in the effects of apple phytochemical extracts. This experiment would be especially worthwhile in light of the fact that Saul *et al.* have reported that quercetin, one of the flavonoid components of apples can activate DAF-16 and cause it to translocate into the nucleus (160).

My findings point to two potential mechanisms, mitigation of oxidative stress and generalized cellular protection, to slow aging, and improve

healthspan and stress resistance. However, it is likely that other mechanisms are present. To get a global view of the anti-aging and other potential effects of apple phytochemicals on *C. elegans*, it would be useful to carry out a microarray experiment at different time points to identify all the genes that are up and down regulated. In addition, it would be interesting to test other flavonoid compounds that are found in apples and to see if they synergize to delay aging.

In conclusion, I have shown that beyond their known healthful properties such as high contents of Vitamin C, and pectin, which has been shown to lower cholesterol, apples also have anti-aging benefits *in vivo*, in the *C. elegans* model. My results implicate the antioxidant and cellular stress resistance pathways as two potential modes of action. However, given recent findings in the field of free radical biology, it is important to keep in mind that antioxidants are not a panacea for slowing down the aging process and increasing lifespan. Moreover, the function of antioxidants is not to remove oxidants entirely, but instead to keep them at an optimum level. Therefore, the anti-aging and stress resistance mechanisms of apple phytochemicals warrant further study.

APPENDIX I

Effects of single phytochemicals quercetin-3-β-D-glucoside and 2-α-ursolic acid found in apples on the lifespan of *C. elegans.*

Many of the health benefits of fruits and vegetables have been proposed to be due to the synergistic effects of the phytochemicals they contain (4). To this end, I screened two individual phytochemicals found in apples, quercetin-3-β-D-glucoside (Figure I.1) and 2-α-ursolic acid (Figure I.2). These compounds have been isolated from the apple peels using bio-guided fractionation (40). I find that at some of the doses tested these compounds still extend lifespan, but to a lesser extent than whole apple phytochemical extracts (Figure I.3). Moreover, in the case of 2-α-ursolic acid, the middle and high doses showed a toxic effect on lifespan. Therefore, it appears that there is an optimum range of doses that can elicit beneficial effects, and there is a reversal of beneficial effects at higher doses. The fact that each single phytochemical extends lifespan to a lesser degree than does the whole apple extract suggests that a mixture of phytochemicals may be necessary to elicit the greatest health benefit. This result is in agreement with previous reports in the literature, which suggest that phytochemicals possess synergistic effects (4).

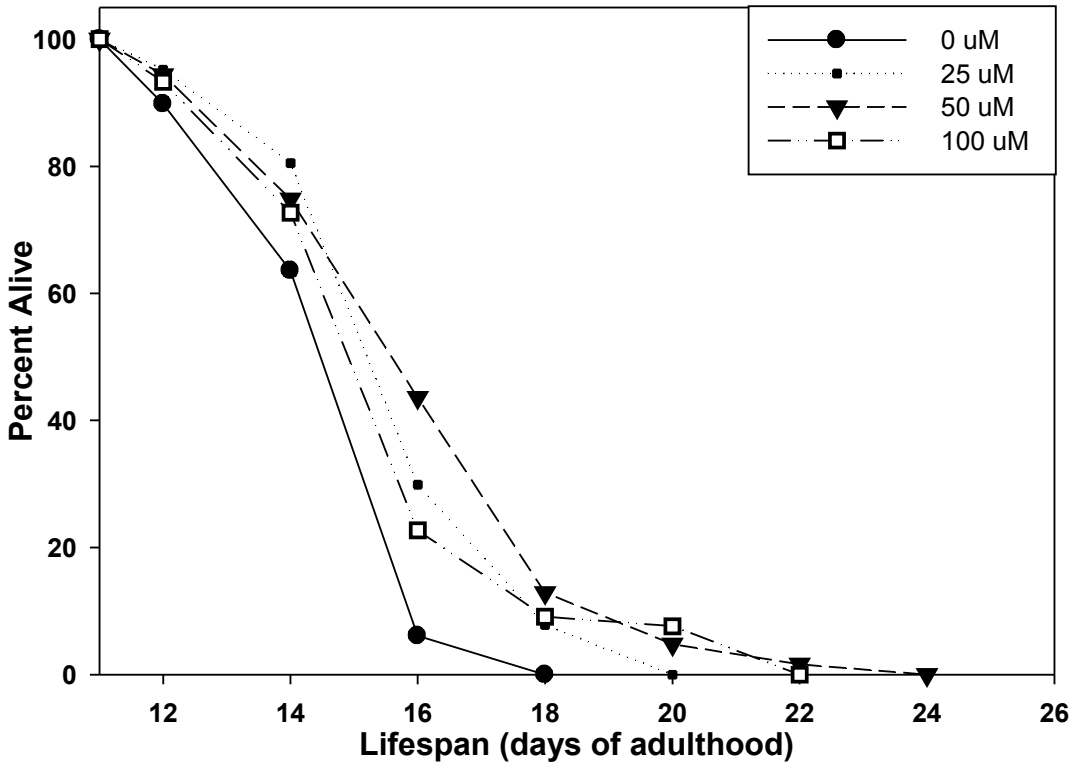


Figure I.1 Effect of quercetin-3-β-D-glucoside on the lifespan of *C. elegans*. Day 1 young adult wild-type animals were treated without (0 µM) or with 25 µM, 50 µM or 100 µM of quercetin-3-β-D-glucoside dissolved in .1% DMSO (see Section 2.2.5 for methods). Survival was monitored starting on day 1 of adulthood. Nematodes, that were exposed to the low and middle dose of quercetin-3-β-D-glucoside survived significantly longer than the control (Table I.1). The experiment was repeated multiple times and a representative trial is shown.

Table I.1 Effect of quercetin-3-β-D-glucoside on mean lifespan of *C. elegans.*

Treatment	N	Mean Lifespan* (days)	Δ**	*p*-value vs. control	% of Control
0 μM	89	15.03±.17	a	N.A.	100.0
25 μM	78	16.23±.21	b	<0.001	108.0
50 μM	69	16.35±.30	b	<0.001	108.8
100 μM	71	15.92±.28	b	<0.01	105.9

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

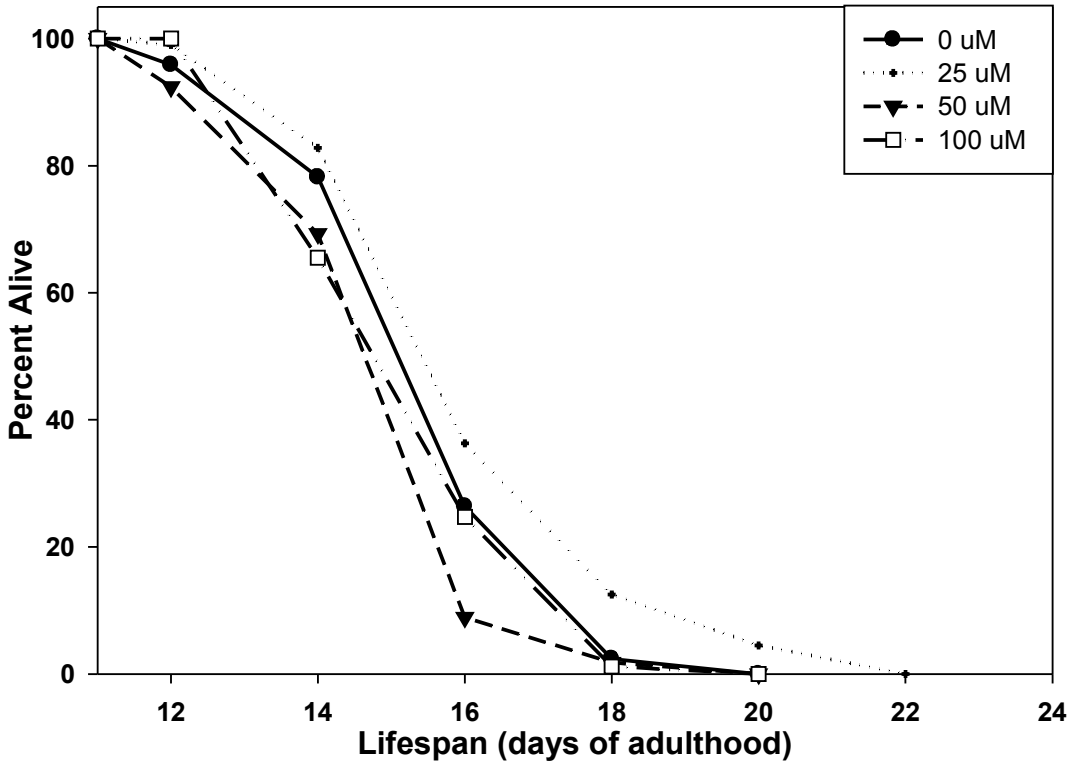


Figure I.2 Effect of 2-α-ursolic acid on the lifespan of *C. elegans*. Day 1 young adult wild-type animals were treated without (0 µM) or with 25 µM, 50 µM or 100 µM of 2-α-ursolic acid dissolved in .1% DMSO (see Section 2.2.5 for methods). Survival was monitored starting on day 1 of adulthood. Nematodes, that were exposed to the low and middle dose of 2-α-ursolic acid survived significantly longer than the control (Table I.1). The experiment was repeated multiple times and a representative trial is shown.

Table I.2 Effect of 2-α-ursolic acid on mean lifespan of *C. elegans.*

Treatment	N	Mean Lifespan* (days)	Δ**	*p*-value vs. control	% of Control
0 μM	98	16.06±.17	a	N.A.	100.0
25 μM	96	16.70±.22	c	0.023	104.0
50 μM	66	15.45±.20	c	0.020	96.2
100 μM	90	15.83±.10	c	0.283	98.6

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

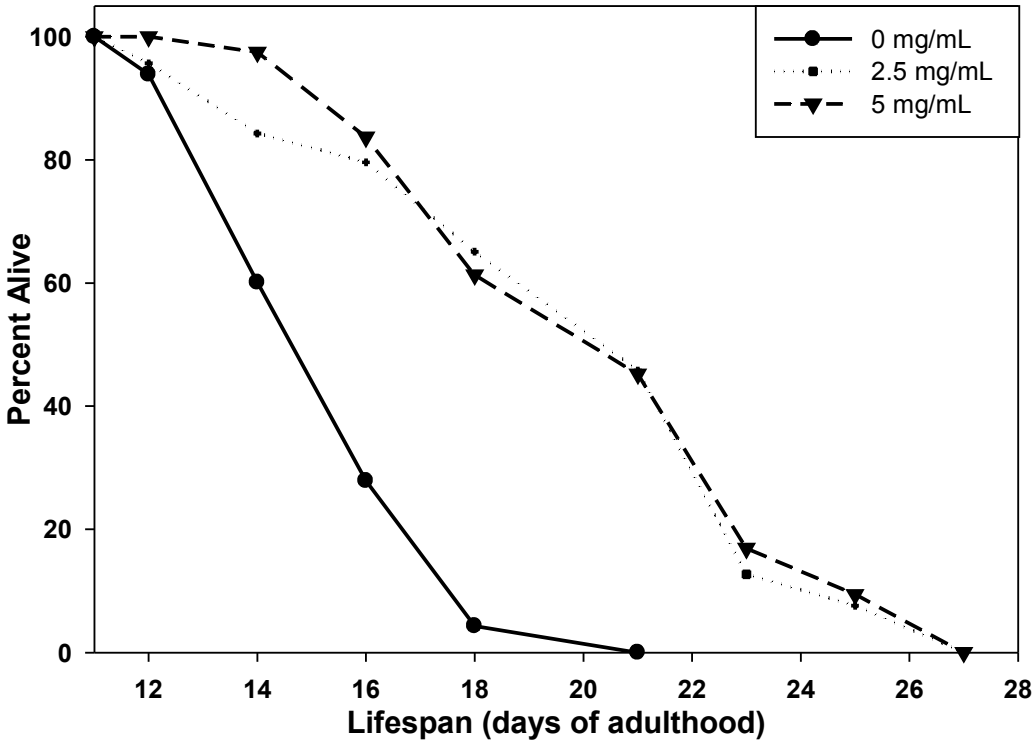


Figure I.3 Effect of apple extracts on the lifespan of N2 wild-type *C. elegans* done in parallel to lifespan experiments with quercetin-3-β-D-glucoside and 2-α-ursolic acid. Day 1 young adult wild-type were treated without (0 mg/mL) or with a low (2.5 mg/mL) and moderate (5 mg/mL) dose of standardized apple extracts, which contained 170 ± 4.6 mg of phenolics per 100 g of apples. Survival was monitored starting on day 1 of adulthood. Nematodes, that were exposed to two levels of apple extracts survived significantly longer than those that did not (p<0.001 for 0 mg/mL compared to each treatment group; log-rank test; see Table I.3) The experiment was repeated multiple times and a representative trial is shown.

Table I.3 Effect of apple extracts on the mean lifespan of N2 wild-type *C. elegans* done in parallel with quercetin-3-β-D-glucoside and 2-α-ursolic acid.

Treatment	N	Mean Lifespan* (days)	Δ**	*p*-value vs. control	% of Control
0 mg/mL	50	15.768±.29	a	N.A.	100.0
2.5 mg/mL	41	20.47±.62	b	<0.001	108.0
5 mg/mL	61	20.894±.43	b	<0.001	108.8

***** Mean ± SEM based upon Kaplan-Meier estimator
****** Values with no letters in common within each column are significantly different; a *p*-value of < 0.05 was considered to be statistically significant based on chi-squared log-rank test).

Appendix II

Additional statistical analysis of One-way ANOVA experiments (Minitab).

II.1 Attenuation of Lipofuscin

```
Factor  Type   Levels  Values
Conc    fixed       4  0.0, 2.5, 5.0, 16.0


Analysis of Variance for Pump, using Adjusted SS for Tests

Source  DF     Seq SS       Adj SS       Adj MS      F      P
Conc     3  3.69202E+20  3.69202E+20  1.23067E+20  59.41  0.000
Error   80  1.65733E+20  1.65733E+20  2.07166E+18
Total   83  5.34935E+20

S = 1439327604   R-Sq = 69.02%   R-Sq(adj) = 67.86%

Tukey Simultaneous Tests
Response Variable Pump
All Pairwise Comparisons among Levels of Conc
Conc =  0.0  subtracted from:

       Difference       SE of           Adjusted
Conc     of Means  Difference  T-Value   P-Value
 2.5  -3481810051   441329610   -7.889    0.0000
 5.0  -2860427947   462323595   -6.187    0.0000
daf-16 1647724508   473420940    3.480    0.0044


Conc =  2.5  subtracted from:

      Difference       SE of           Adjusted
Conc    of Means  Difference  T-Value   P-Value
 5.0   621382105   422291424    1.471    0.4594
daf-16 5129534559   434412624   11.808    0.0000


Conc =  5.0  subtracted from:

      Difference       SE of           Adjusted
Conc    of Means  Difference  T-Value   P-Value
```

daf-16 4508152455 455725366 9.892 0.0000

II.2 Survival of *C. elegans* after 35°C Heat Shock

One-way ANOVA: % Alive versus Strain-or-Conc

Source	DF	SS	MS	F	P
Strain-or-Conc	5	18643.4	3728.7	102.83	0.000
Error	27	979.0	36.3		
Total	32	19622.4			

S = 6.022 R-Sq = 95.01% R-Sq(adj) = 94.09%

Level is either N2 on 0 mg/mL or indicated concentration of apple extract or indicated strain on 0 mg/mL

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
0.0	3	55.71	1.24	(---*--)
10.0	6	96.13	4.78	(-*--)
2.5	6	71.40	8.07	(--*-)
5.0	6	87.17	6.22	(--*-)
age-1	6	89.73	5.34	(--*-)
daf-16	6	28.96	6.33	(-*--)

40 60 80 100

Tukey Simultaneous Tests
Response Variable Fluor
All Pairwise Comparisons among Levels of Conc
Conc = 0.0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
age-1	40.42	4.258	9.492	0.0000
2.5	15.68	4.258	3.683	0.0117
5.0	31.46	4.258	7.389	0.0000
10.0	34.02	4.258	7.990	0.0000
daf-16	-26.75	4.258	-6.283	0.0000

Conc = age-1 subtracted from:

Difference	SE of	Adjusted

Conc	of Means	Difference	T-Value	P-Value
2.5	-24.74	3.477	-7.12	0.0000
5.0	-8.96	3.477	-2.58	0.1379
10.0	-6.40	3.477	-1.84	0.4582
16.0	-67.17	3.477	-19.32	0.0000

Conc = 2.5 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
5.0	15.78	3.477	4.54	0.0013
10.0	18.34	3.477	5.27	0.0002
daf-16	-42.43	3.477	-12.21	0.0000

Conc = 5.0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
10.0	2.56	3.477	0.74	0.9755
daf-16	-58.21	3.477	-16.74	0.0000

Conc = 10.0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
daf-16	-60.77	3.477	-17.48	0.0000

II.3 Induction of SOD-3 promoter in SOD-3::GFP worms

Factor	Type	Levels	Values
Conc	fixed	4	0.0, 2.5, 5.0, 10.0

Analysis of Variance for Fluor, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Conc	3	14.5240	14.5240	4.8413	12.86	0.000
Error	53	19.9596	19.9596	0.3766		
Total	56	34.4837				

S = 0.613675   R-Sq = 42.12%   R-Sq(adj) = 38.84%

Tukey Simultaneous Tests
Response Variable Fluor
All Pairwise Comparisons among Levels of Conc
Conc = 0.0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
2.5	-1.211	0.2187	-5.537	0.0000
5.0	-1.235	0.3392	-3.642	0.0034
10.0	-0.910	0.1971	-4.616	0.0002

Conc = 2.5 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
5.0	-0.02463	0.3479	-0.07080	0.9999
10.0	0.30100	0.2117	1.42155	0.4918

Conc = 5.0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
10.0	0.3256	0.3348	0.9726	0.7655

II.4 Induction of GST-4 promoter in GST-4::GFP worms

Factor	Type	Levels	Values
Conc	fixed	4	0.0, 2.5, 5.0, 10.0

Analysis of Variance for Fluor, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Conc	3	6.4708	6.4708	2.1569	6.37	0.001
Error	111	37.5665	37.5665	0.3384		
Total	114	44.0372				

S = 0.581753   R-Sq = 14.69%   R-Sq(adj) = 12.39%

Tukey Simultaneous Tests
Response Variable Fluor
All Pairwise Comparisons among Levels of Conc
Conc = 0.0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
2.5	-0.1294	0.1510	-0.857	0.8267
5.0	-0.5421	0.1495	-3.627	0.0024
10.0	-0.5034	0.1510	-3.335	0.0063

Conc = 2.5 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
5.0	-0.4127	0.1569	-2.630	0.0473
10.0	-0.3740	0.1583	-2.362	0.0906

Conc = 5.0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
10.0	0.03868	0.1569	0.2465	0.9947

II.5 Induction of Hsp16.2 promoter in Hsp16.2::GFP worms

Factor	Type	Levels	Values
Conc	fixed	2	0, 10

Analysis of Variance for Fluor, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Conc	1	1688.8	1688.8	1688.8	12.98	0.002
Error	19	2471.3	2471.3	130.1		
Total	20	4160.1				

S = 11.4048 R-Sq = 40.59% R-Sq(adj) = 37.47%

Tukey Simultaneous Tests
Response Variable Fluor

All Pairwise Comparisons among Levels of Conc
Conc = 0 subtracted from:

Conc	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
10	18.12	5.029	3.603	0.0019

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